The regulatory effect of melatonin on molecular changes induced by radiation and chemotherapy in breast cancer cells

Master's Final Project

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June 2021

Master's in Molecular Biology and Biomedicine

Universidad de Cantabria and Universidad del País Vasco/Euskal Herrico Unibertsitatea

ÍNDICE

INTRODUCTION

Breast cancer

Breast cancer (BC) is a widespread, heterogenous and complex neoplastic disease that affects nearly one of eight women during their lifetime. It is characterized by an abnormal cell proliferation within the mammary glands, changes in cell genetic material and a polarization of cellular behaviour towards a malignant stage. In advanced stages, cells can disseminate from the primary focus and invade different tissues via bloodstream or lymphatic system, starting a process known as metastasis (American Cancer Society, 2021; Akram *et al.*, 2017; Martínez-Campa *et al.*, 2017).

Metastatic processes start when a primary solid tumour invades the adjacent tissue and spreads to the neighbouring, reaching distant organs. Early stages of metastasis strongly rely on epithelial to mesenchymal transition (EMT), following extravasation, colonization and differentiation. Therefore, epithelial cells are the major cellular type involved in tumorigenic processes, generating carcinomas. In spite of this, muscle cells and myoepithelial cells can generate tumours, sarcomas and myoepithelial carcinomas respectively, although this happens with a lesser frequency (American Cancer Society, 2021; Nurzadeh *et al.*, 2021; Akram *et al.*, 2017).

Attending to its histological and molecular characteristics, breast cancer can be divided into different groups (Akram *et al.*, 2017). Commonly, breast cancer is categorized considering distinctive molecular and phenotypical features:

- Breast cancer expressing hormonal receptors: estrogen receptor (ER+) and progesterone receptor (PR+).
- Breast cancer expressing human epidermal growth receptor 2 (HER2+).
- Triple negative breast cancer (TNBC): tumours which do not express any receptor.

1. Epidemiology

Breast cancer is the most common cancer in women and consequently, the primary cause of mortality due to cancer in female, with 1.2 million of new cases being diagnosed and 500000 associated deaths per year. In Spain, 5-year survival expectancy is close to 85%, calculating 25000 annual new cases, an incidence that is showing a progressive growth in the last decades. Most cases are diagnosed in patients between 35 and 80 years old (Martín *et al.*, 2015).

Breast cancer incidence is tightly related to socioeconomic situation. As a result, higher breast cancer rates are associated to western countries in USA and Europe. Accordingly, Asia and Africa registered lower incidence rates, especially in underdeveloped countries, as shown in **Figure 1** (Martín *et al.*, 2015). In spite of this, general advances in diagnosis and treatment of this pathology, combined with promising new approaches based on personalized medicine, can serve as the foundation to reduce breast cancer incidence.

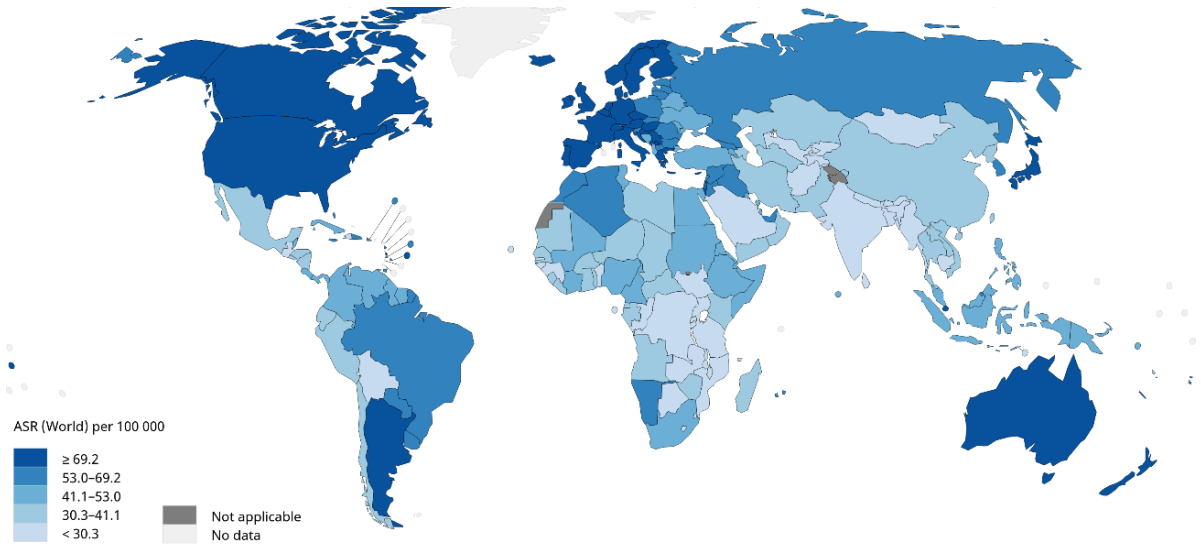


Figure 1. Breast cancer worldwide incidence. *Estimated age-standardized incidence rates in 2020. Source: GLOBOCAN (2021, online).*

2. Etiology

The precise mechanisms that trigger breast cancer onset are still unknown. However, there is a vast list of clinical and genetic factors associated to this pathology that can impact directly or indirectly into the development of the disease. These factors can be classified into modifiable (endogenous estrogens levels, obesity, hormone therapy, alcohol consumption, etc.) and non-modifiable (gene mutations, family and reproductive history, age, etc.). However, there are some controversial opinions regarding whether some factors are modifiable or not (Barzaman *et al.*, 2020; Martín *et al.*, 2015; Samavat & Kurzer, 2015). Hiatt *et al.* (2020) proposed a conceptual model of breast cancer etiology, illustrated in **Figure 2**.

One important factor is age, which can be in turn related to other factors. Age increases breast cancer incidence progressively, until the range of 40-50 years, with a following stabilization. Survival rate also decreases to the growing age. Socioeconomic level has also an important impact, as stated before, affecting women with a higher educational, economical and occupational level, especially the ones residing in urban areas. Nevertheless, this is a challenging factor to measure. Lifestyle is another factor to take into consideration, being primarily important the association of breast cancer with sedentarism and overweight, highly related with diet. At the same time, alcohol intake can raise the level of estrogen related hormones by stimulating estrogen receptor pathways and have a negative impact in breast cancer pathology (Hiatt *et al.*, 2020; Naeem *et al.*, 2019; Martín *et al.*, 2015).

Breast cancer progression can be favoured by hereditary factors as the presence of family members with breast or ovary cancer. In this way, the closer the kinship or the earlier the first case, the greater probability of developing breast cancer. One third of patients reporting a family case of breast cancer are carriers of an inheritable mutation in *BRCA1* or *BRCA2*, *HER2*, *PIKC3A* genes. These are important genes because they play a functional role in homologous recombination and DNA damage. Healthy women carrying a

mutation in *BRCA1* and *BRCA2* genes increase up to a 60% the probability of developing breast cancer in the long term (Naeem *et al.*, 2019; Martín *et al.*, 2015).

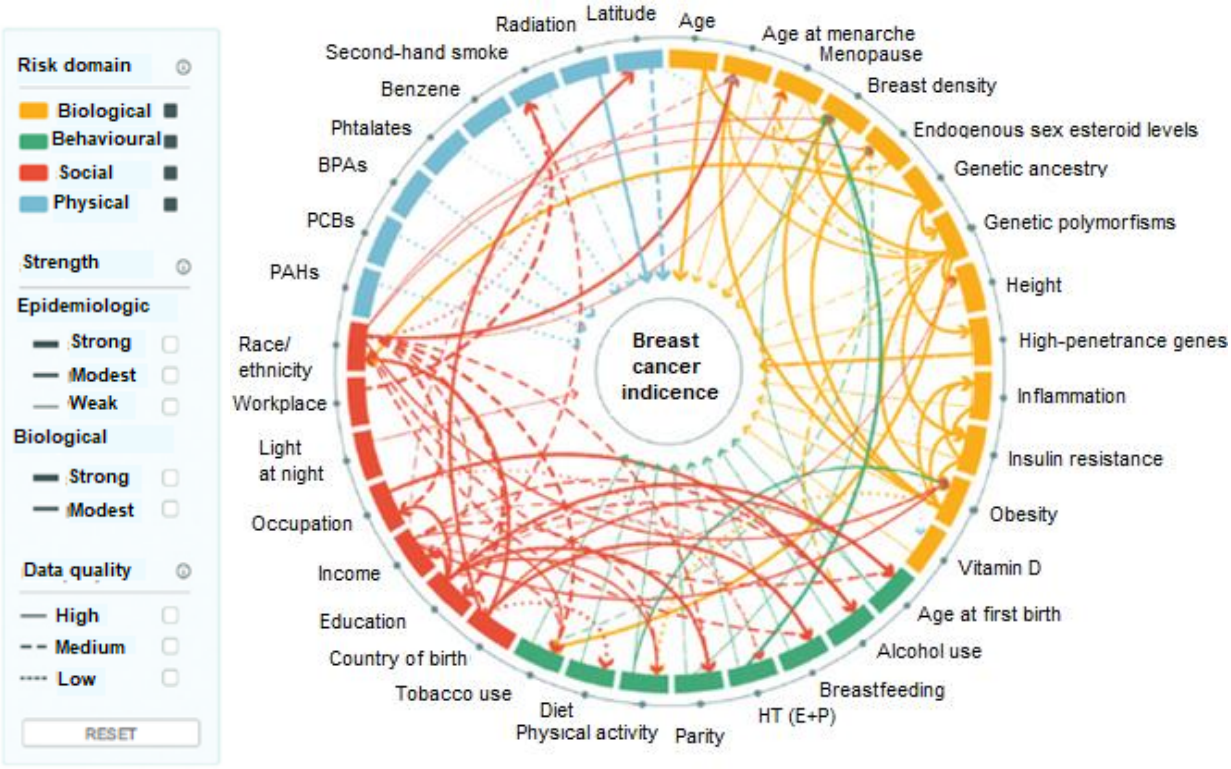


Figure 2. ***Conceptual model of breast cancer etiology.*** *Causative factors are displayed in four domains: social (orange), physical (blue), biological (yellow) and behavioral (green). The thickness of arrows indicates strength of the association, as well as quality of data indicated as solid, broken or dotted arrows. The greater strength and quality of data is pictured as a thick solid arrow. Source: Hiatt* et al. *(2020).*

Mammary lesions as sclerosing adenosis, intraductal papilloma, ductal atypical hyperplasia, etc. strongly increment the risk of developing breast cancer. Regarding this, hormonal treatment can also result in this pathology. For example, prolonged exposure to high estrogen levels, both endogenous or exogenous, can influence breast cancer development, that may be the case of late menopause, premature menarche or women that have not given birth (Naeem *et al.*, 2019; Martín *et al.*, 2015).

These biological, behavioural, social and physical relationships, demonstrate the complexity of breast cancer etiology. The study of these causative factors and interconnections between them, can help to stratify patients with higher predisposition to develop the disease and be further supervised by specialists, substantially increasing life expectancy.

3. Estrogen role in breast cancer

Estrogen implication in breast cancer development was hypothesized when bilateral oophorectomy was associated to a significantly reduced breast cancer risk. This reduction was even greater when ovaries were removed at an early age. This added to the fact that 2/3 of breast cancer tumours are ER+ and respond to circulating estrogen levels, sheds a light into the importance of deciphering the estrogen-dependent mechanisms involved in breast cancer progression (Samavat & Kurzer, 2015).

As stated before, estrogen levels can influence breast cancer onset and associated risk. Epidemiological studies have consistently shown a 2–3-fold increase in breast cancer risk in women with elevated blood estrogen levels. The most common circulating estrogens are estradiol, estrone and estriol. Estradiol is secreted by ovarian granulosa cells and is the most biologically active estrogen form. Estradiol can also be formed by estrone conversion through 17β-hydroxysteroid dehydrogenase enzyme. Ovarian theca cells can secret androstenedione during the menstrual cycle, which acts as a precursor of estrone and testosterone. This last hormone can also be converted into estradiol by aromatase enzyme in peripheral tissues, highly important in post-menopausal woman due to cessation of follicular function (Samavat & Kurzer, 2015).

Estrogens play a major role in promoting breast epithelium proliferation in both normal and neoplastic tissues. Thus, they have been considered as breast carcinogens due to their participation in three principal processes: hormonal activity-mediated cell proliferation (1), genotoxic effects by the increase of mutation rates derived from cytochrome P450 metabolic activity (2) and aneuploidy induction (3) (Russo & Russo, 2006).

In mouse models, tumorigenic effects of estrogens are associated with hyperprolactinemia and pituitary hyperplasia increasing the number of hyperplastic prolactin-secreting cells. Estrogen binding to nuclear receptor alpha (ER-α) is the main mechanism involved in carcinogenicity, entailing a potent stimulus on breast cancer cell proliferation through growth factor production. Moreover, several studies have claimed that specific estrogen metabolites might generate several DNA mutations that may trigger breast cancer onset (Russo & Russo, 2006).

4. Breast cancer treatment

4.1. Anti-estrogenic therapy (hormonotherapy)

One of the main goals in the treatment of breast cancer is to neutralize the effects of estrogens on the breast, due to their involvement in breast cancer progression, especially in breast cancer expressing hormone receptors (Barzaman *et al.*, 2020; Russo & Russo, 2006). The pharmacological strategies employed to selectively neutralize the effects of estrogens on the breast are:

- The use of Selective Estrogen Receptor Modulators (SERMs). These are molecules that bind and block the estrogen receptor (ER), thus preventing estrogen binding. The most common are tamoxifen, fulvestrant and raloxifene. Nevertheless, they can exert their anti-neoplastic function non-specifically in other tissues. For example, tamoxifen can target estrogen in the uterus, liver or bone, leading to osteoporosis, among other malignancies (Barzaman *et al.*, 2020; Akram *et al.*, 2017).
- The use of Selective Estrogen Enzyme Modulators (SEEMs). These molecules inhibit enzymes involved in the synthesis of steroid hormones (therefore preventing estrogen synthesis) or activate of enzymes related with estrogen inhibition. Aromatase inhibitors are an important

example of this molecules, preventing ovary hormone production. This is the case of letrozole, anastrozole and exemestane (Barzaman *et al.*, 2020).

Toxic effects of anti-estrogenic therapy are not as significant as those linked to other cytotoxic drugs, although there are some side effects associated to this treatment. Even though, medical indications for discontinuing the use of this treatment include more severe affections (Akram *et al.*, 2017).

4.2. Chemotherapy

Chemotherapy is traditionally one of the most common breast cancer treatments, especially in aggressive subtypes. Chemotherapeutic agents include: anthracyclines (epirubicin, doxorubicin), taxanes (paclitaxel, docetaxel), capecitabine, vinorelbine, platinum drugs (cisplatin, carboplatin), gemcitabine, trastuzumab and angiogenesis inhibitors. However, chemotherapy has devastating side effects, mainly caused by the unspecific targeting, that may attack all growing cells including immune cells, therefore decreasing immunological responses. At the tumour microenvironment level, chemotherapeutic agents have positive effects, helping immune cells to eradicate or supress tumour growth by selectively polarizing immune cell population (Barzaman *et al.*, 2020).

In this work, we will test two common chemotherapeutic drugs: docetaxel and doxorubicin. The first one is a taxane that acts stabilizing microtubules through tubulin binding, consequently preventing cell division. The second one is an anthracycline that works by intercalation between double DNA helix, inhibiting DNA dependent DNase and RNA polymerases. This consequently interrupts DNA replication, therefore doxorubicin has a stronger effect on the S phase of cell cycle. Rupture of DNA strands is associated with formation of free radicals, responsible for the inhibition of respiratory chain enzymes in mitochondria and membrane lipid oxidation (Saloustros *et al.*, 2008; Czeczuga-Semeniuk *et al.*, 2004).

A correct chemotherapeutic dosage is needed for effective treatment, as high doses could completely supress immune responses. The search of an adequate chemotherapeutic dose with minimal side effects led to metronomic chemotherapy (MTC). This treatment is based on regular and low dosage administration of chemotherapeutic agents, being specifically important in treating angiogenesis and vasculogenesis. It can also restore anti-tumoral responses within the tumour microenvironment, specifically targeting Treg cells (Barzaman *et al.*, 2020).

Chemotherapeutic agents can also promote immune cell death (ICD), mainly mediated by calreticulin. This is why the synergistic effect of chemotherapeutic and immunotherapy can enhance treatment effectiveness. As an example, according to Barzaman *et al.* (2020), it was observed that chemotherapy in combination with trastuzumab could increase breast cancer patients survival in a 35%. What is more, antigens released during chemotherapy within the tumour microenvironment, could be helpful in immunotherapeutic strategies. Chemotherapeutic drugs used in this project (docetaxel and doxorubicin), can both increase the number of T and NK cells and reduce B cells or activate NK cells in the case of docetaxel (Barzaman *et al.*, 2020).

4.3. Radiotherapy

Radiation therapy (RT) is reported to be effective especially in early stages of breast cancer and is commonly used in combination with breast conserving surgery. As radiation kills malignant cancer cells, radiation is used to eliminate reminiscent cells in the tumoral area (Akram *et al.,* 2017). According to Piroth *et al.* (2019), whole-breast irradiation with a total dose of 50 Gy can reduce local recurrence rate by 70-88%. However, these techniques can increase cardiac toxicity, especially in left-sided breast cancer patients, leading to radiation-induced heart disease (RIHD) (Piroth *et al.*, 2019).

Nevertheless, total dosage was significantly reduced over the past few years thanks to the application of new techniques as three-dimensional treatment planning, optimizing the treatment according to patient's anatomy. For example, average dose in 1970s was 13.3 Gy, however, in 2007 main dosage was reduced to 2.3 Gy. These significantly diminished therapy complications as skin desquamation, edema, late fibrosis, etc. (Piroth *et al.*, 2019; Mcdonald *et al.*, 2016).

Role of microRNAs in breast cancer

MicroRNAs, commonly known as miRNAs, are post-transcriptional gene regulators that participate in normal and disease-associated cellular processes. Therefore, these non-coding RNA molecules are a fundamental basis to study the etiopathogenesis of several human diseases, being cancer one of them (Singh & Mo, 2013).

miRNAs are single-stranded, non-protein coding, conserved, endogenous molecules, ranging from 19 to 25 nucleotides in length. It is thought that the human genome contains 2654 mature miRNAs, constituting 1-2% of eukaryotic genome. These molecules coordinate multiple pathways involving gene activation or more frequently, gene repression. According to Loh *et al.* (2019) it is believed that they could regulate one-third of protein coding genes. They exert their function by degradation (if there is perfect complementarity between miRNA and mRNA sequences) and translational repression (if there is partial complementarity), binding to the target mRNAs (messenger RNA). What is more, one single mRNA can be cooperatively regulated and targeted by multiple miRNAs, amplifying their impact (Loh *et al.*, 2019; Singh & Mo, 2013).

The biogenesis of mature miRNA involves a series of biological processes gathered in **Figure 3A**. The process starts by a primary miRNA transcript (pri-miRNA), that is transcribed in the nucleus by RNA polymerase II/III. The resulting molecules are nucleotide-based structures with local stem loops, a 5' cap and a poly-A tail. Pri-miRNA is processed by Drosha (RNAse III-type endonuclease) into a 70-nt hairpin structure known as precursor miRNA (pre-miRNA). This pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5 RanGTP-dependent nuclear transport and loaded onto Dicer. This RNAse III enzyme, thanks to the aid of transactivation response RNA binding protein (TRBP), generates a double-stranded structure formed by mature miRNA/antisense miRNA duplex. The latter is usually degraded, whereas the long mature miRNA strand is incorporated into the miRNA-induced silencing complex

(RISC) leading to gene silencing. Gene silencing mechanism of action depends on mature miRNA binding to its seed sequence (2-8 nucleotides from the 5'- end) to the 3'-UTR (or 5'-UTR and open reading frame (ORF)) region of the target mRNA (Loh *et al.*, 2019; Hamam *et al.*, 2017).

miRNAs participate in different breast cancer cellular pathways (proliferation, apoptosis, metastasis, cancer recurrence and chemoresistance, among others), considered the hallmarks of cancer disease. Depending on their overall action, they can be classified into oncogenic (oncomiR) or tumour suppressor (tsmiR), targeting tumour suppressor genes or oncogenes, respectively (Nurzadeh *et al.*, 2021; Loh *et al.*, 2019) (**Figure 3B**).

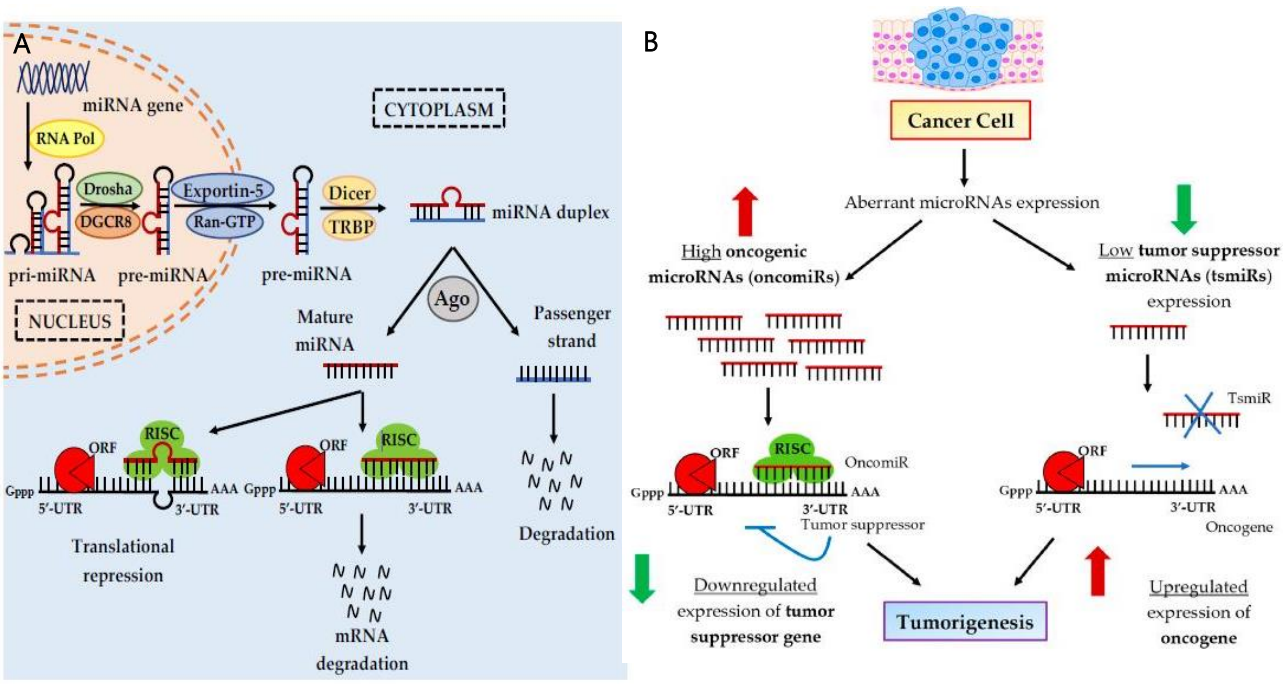


Figure 3.* (A) MicroRNA biogenesis and modulation of miRNA activity.** *miRNA genes are transcribed to produce primary miRNA transcripts. Subsequently, Drosha cleaves the pri-miRNA into a precursor miRNA transcript (pre-miRNA) then transported into the cytoplasm via nuclear pore by exportin 5. In the cytoplasm, pre-miRNA is modified by the Dicer and TRBP complex to form a mature miRNA duplex. The miRNA duplex is unwound by helicase into two single-stranded miRNAs and incorporated into RISC complex. This complex can then bind to target mRNA and exert its inhibitory function through translational block or miRNA degradation.* ***(B) Regulatory mechanisms of miRNAs in tumorigenesis. *Within the tumoral microenvironment, cancer cells undergo high expression of oncomiRs and low expression of tsmiRs. This leads to inhibition of tumour suppressor genes and enhancement of oncogene expression, respectively, which favors tumoral processes. Source: Loh* et al. *(2019).*

1. Regulatory role of miRNAs involved in breast cancer

Few studies have evaluated different patterns of miRNA expression in breast cancer patients. Establishing the main pathways related to these changes in expression and how to regulate them, has become the main focus on novel breast cancer miRNA-therapy. As a general overview, there are some miRNAs that are significantly dysregulated in breast cancer (miR-125b, miR-145, miR-21 and miR-155) (Singh *et al.*, 2013). In this work, we are going to cite the most important miRNAs involved in breast cancer pathology and relate them to the hallmarks of the disease.

1.1. Cell proliferation and cell cycle regulation

Cell proliferation is the principal process involved in breast cancer development and is tightly regulated by cell cycle progression. Cyclin E1 is an important regulator of G_1-S cell cycle transition and is the target of tsmiRs miR-497, miR-483 and miR-16. Overexpression of this miRNAs inhibited cell proliferation and cell cycle progression, preventing DNA synthesis during S-phase (Loh *et al.*, 2019).

miR-221/222 cluster overexpression was demonstrated to be involved in S phase initiation and induction of cell proliferation. Its principal targets are cell cycle inhibitors like p27 and p57, resulting in Wnt, Notch and MAPK signalling pathways promotion (Nurzadeh *et al.*, 2021).

miR-106A/363 cluster is a paralogue of miR-17/92 and encodes for miR-106a, miR-18b, miR-20b, miR-19b, miR-92a and miR-363. The cluster was related with PI3K/AKT pathway and associated to tumour size and lymph node metastasis. Specifically, this miRNA cluster regulates *PTEN*, inhibiting its expression, consequently increasing cell growth, proliferation and metastasis through DNA synthesis (Nurzadeh *et al.*, 2020; Fang *et al.*, 2017).

Breast cancer cell viability can also be increased by upregulation of some protein kinases and downregulation of its inhibitors. miR-143 and miR-455 expression counteracts the proliferative effects of cyclin D1, MAP3K7 (mitogen-activated protein kinase) and CDK14 (Cdc2-related protein kinase). miR-424 overexpression allows the inhibition of cellular proliferation by arresting cells in the G2-M phase. This anti-oncogenic function of miR-424 is dependent on its binding to *CDK1* (Loh *et al.*, 2019).

1.2. Invasion and metastasis

Invasiveness and metastatic potential of cancerous cells lies on their poor adhesion to extracellular matrix and consequent loss of cell-matrix interactions. This is happens because of EMT process, characterized by the loss of E-cadherin and switch to N-cadherin, reducing cell localization and interactions, allowing them to invade and metastasize distant organs via blood or lymphatic systems (Loh *et al.*, 2019). There are many miRs related to these processes. Thus, overexpression of miR-200c/141 cluster in breast cancer cells is related to increased *C-JUN, C-FOS* and *FOSB* mRNAs, upregulating the expression of Serpine B2, a mediator of cell metastasis in the lungs and lymph nodes (Nurzadeh *et al.*, 2021; Loh *et al.*, 2019). miR-10b and miR-221/222 cluster are also responsible for inducing EMT by targeting *TWIST-1* gene (miR-10b) or suppressing Notch3 (Nurzadeh *et al.*, 2021; Loh *et al.*, 2019).

Wnt/β-catenin signalling pathway was shown to be related to metastatic processes in breast cancer, regulating EMT. miR-148a, a target for WNT-1 (a negative regulator of Wnt/β-catenin pathway) is downregulated in cancerous processes and related to an increase in migration and invasion capacity. Additionally, miR-340 is thought to inhibit migration, invasion and metastasis by also targeting this particular pathway. Moreover, miR-29a is also related to EMT and metastasis, specifically promoting these features through tristetraprolin targeting (Singh & Mo, 2013).

On the other hand, miR-497 and miR-204 had a suppressive role on cell proliferation and migration, specifically supressing *SMAD7* gene expression in the case of miR-497. As a consequence, immune response and tumour immune escape are inhibited (Loh *et al.*, 2019).

Finally, miR-17/92 cluster is also involved in invasion and metastasis, however it was reported that miR-17 could affect differentially depending on the breast cancer subtype implicated. In some cases, it can supress c-Myc through ROCK signalling pathway and PTEN suppression, leading to the activation of Wnt/β-catenin pathway. However, in other cases it can inhibit cell proliferation through *ETV1* suppression, acting as a tumour suppressor (Nurzadeh *et al*., 2021).

1.3. Apoptotic response and cell death

Apoptosis is a defence mechanism induced by alterations in chromosomal or genetic content of normal cells. This means cell viability can be increased if some point of the apoptotic process is disrupted. Cancer cells evade apoptotic response through multiple mechanisms: loss of tumour suppressor proteins, dysregulation of caspase activity, upregulation of pro-survival regulators, downregulation of pro-apoptotic factors and deactivation of death ligands (Loh *et al.*, 2019).

For instance, miR-204 overexpression promotes apoptosis by targeting *JAK2* and decreasing anti-apoptotic proteins like Bcl-2 and survivin. miR-148a overexpression also reduces cell viability and chemoresistance by supressing *BCL-2*, its main target (Loh *et al.*, 2019).

miR-21 and miR-203 are oncogenic miRNAs targeted by an endogenous protein known as kallistatin. This protein induces cell death by activating caspase-3 in breast cancer cells. Kallistatin inhibited miR-21 function via AKT pathway, reducing the expression of *BCL-2*. This protein also inhibited miR-203 expression levels via PKC-ERK activation increasing tumour suppressor SOCS3 (Nurzadeh *et al*., 2021; Loh *et al.*, 2019).

Another aspect to take into consideration is cell immortality due to telomerase activity. miR-155 is related to telomere protection by negatively targeting *TRF1*, resulting in genomic instability. As a result, miR-155 is associated with poor clinical outcomes in some types of breast cancer. However, in other studies, miR-155 is associated with suppression of apoptotic processes by inducing cell cycle arrest and p53-mediated apoptosis (Nurzadeh *et al.*, 2021; Loh *et al.*, 2019).

1.4. Hypoxia and angiogenesis

Cellular growth and invasion entail an increase in cellular metabolic activity. As cellular density rises, oxygen concentration decreases significantly in comparison to healthy tissues. This tumour microenvironmental condition, known as hypoxia, is a key regulator of angiogenic processes in breast cancer (Loh *et al.*, 2019). HIF (Hypoxia-induced Factor) and VEGF (Vascular Endothelial Growth Factor) are two major factors that regulate critical processes including cell homeostasis and proliferation, metastasis and angiogenesis (Loh *et al.*, 2019).

miR-210 is one of the most induced miRNAs during hypoxia, associated with *HIF* expression and angiogenesis. Another miRNA related to this process is miR-20a, a member of the miR-17/92 cluster capable of inducing an angiogenic pattern by overexpression of *VEGFA*, linking the cluster to an increase of VEGF levels. Finally, miR-93 overexpression can increase blood vessel formation by targeting β-integrin repression, involved in endothelial cell separation that lead to blood vessel formation (Nurzadeh *et al.*, 2021). Moreover, miR-497 and miR-140 supressed angiogenesis by regulating VEGF and HIF-1α, disrupting the formation of capillary structures and reducing microvascular density (Loh *et al.*, 2019).

Finally, several studies demonstrated that mesenchymal stem cells (MSCs) can be recruited to the tumour microenvironment promoting tumour progression by interacting with tumour cells. MSCs can secret extracellular vesicles shaping the tumoral stroma by transference of their components, including miRNAs. For example, miR-100 is enriched in MSC-derived exosomes. This is believed to be responsible for reducing VEGF expression, acting as a angiogenic suppressor (Loh *et al.*, 2019).

1.5. Resistance to breast cancer therapy

One of the major obstacles in breast cancer therapy is the resistance to conventional therapies, leading to undesirable secondary effects. The key genes involved in breast cancer drug resistance are *MDR1* (multidrug resistance 1), *MRPs* (multidrug resistance-associated proteins), *BCRP* (breast cancer resistance protein), *GSTπ* (glutathione S-transferase π) and DNA repair genes. The first three belong to the ATP-binding cassette (ABC)-superfamily multidrug efflux pumps, whose function is to pump toxic metabolites and xenobiotics out of the cell to protect homeostasis. GSTπ is a detoxification enzyme that mediates the efflux of chemotherapeutic drugs. These key genes can be targeted by miRNAs that modulate their activity. For example, miR-451 and miR-298 regulate MDR1 in doxorubicin resistance (Singh & Mo, 2013).

Another common problem in hormone-dependent breast cancer treatment is resistance to tamoxifen. miR-15a/16 cluster is related to tamoxifen, by increasing cell proliferation and cell cycle progression. This is related to E2F7 factor, correlated with a poor prognosis in tamoxifen-treated patients. E2F7 overexpression induces transcription of miR-15a/16, promoting cyclin E1 and further inducing cell growth (Loh *et al.*, 2019). On the contrary, this effect can be reduced by miR-206 overexpression, thus reducing G_1-S transition by regulating cell cycle associated proteins like p21, CDK4 and cyclin D1 (Loh *et al.*, 2019).

2. MiRNAs as molecular markers for early breast cancer detection

As stated before, miRNAs fulfil their functions through mechanisms of action which are dependent on cellular content, revealing tissue or cell-specific phenotypes. Therefore, miRNome profiling could be an interesting technique to adjust treatments according to molecular features of cancer subtypes. This is why miRNAs have a unique potential to be used as biomarkers for breast cancer detection, clinical outcome and prognosis (Asiaf *et* al., 2018; Singh & Mo, 2013).

More importantly, miRNAs can serve as markers for the distinctive features of breast cancer pathology, as they are related to different mechanisms and molecular characteristics. For example, miR-200c/141 cluster could serve as a prognostic indicator, as it is highly expressed in basal-like cancers. Levels of this cluster in metastatic cancers were also higher compared to localized tumours, having a potential

application as a breast tumour metastasis biomarker as well. MiR-34, miR-10b and miR-155 are also considered metastasis biomarkers (Loh *et al.*, 2019; Asiaf *et al*., 2018).

Several miRNAs can also be a sign of drug resistance. This is the case of miR-200a and miR-210, whose plasma levels are associated to breast cancer stage and chemotherapy resistance. Patients showing high levels of this miRNAs are prone to chemotherapy resistance and possibly are found in an advanced stage of breast cancer. For instance, miR-210 levels positively correlated with brain, lung and liver internal organ metastasis and stage IV breast cancer (Loh *et al.*, 2019).

In spite of their potential application, novel miRNA biomarkers should be validated through clinical and analytical assays, leaning towards a personalized treatment for early diagnosed patients with breast cancer. This needs to be done using prognostic biomarkers in combination with validated prognostic/predictive factors (Barzaman *et al.*, 2020).

3. Therapeutic potential of miRNAs in breast cancer

Due to miRNAs implication in signalling pathways related to breast cancer pathological processes, experts have developed a growing interest into miRNA-based therapies. Targeting specific miRNAs could also serve as a novel strategy to overcome drug resistance and improve therapeutic outcomes, being used as adjuvants for anti-cancer drugs (Nurzadeh *et al*., 2021; Singh & Mo, 2013).

miRNA-based therapies rely on miRNA mimics (replacement or restoration of miRNAs) or antagomiRs (miRNA reduction or suppression). On one hand, miRNA mimics are aimed to restore tumour suppressor normal miRNA levels using RNA duplexes. This can also be achieved by DNA plasmids encoding a concrete miRNA gene. These technologies should be applied considering that: they should enter the cytoplasm, tumour suppressor activities should prevail over oncogenic miRNAs and they should also target the same set of regulator mRNA as the original miRNAs (Nurzadeh *et al.*, 2021; Singh & Mo, 2013).

On the other hand, miRNA inhibition therapy specifically targets miRNA biogenesis or blocks miRNA function. This has been done by cholesterol-conjugated antimiRs (AMOs or ASOs), miRNA sponges, artificial miRNA (amiRNA) and CRISPR/Cas9 genome editing, among other strategies. miRNA sponges are competitive inhibitors fitted with several binding sites to a certain miRNA or miRNA family (Nurzadeh *et al.*, 2021; Asiaf *et al*., 2018).

Nowadays miRNA delivery is based on nanoparticles that rely on materials like polyethylene glycol (PEG), inorganic nanoparticles, lipid carriers, synthetic polyethyleneimine, chitosan, atelocollagen, etc. (Singh & Mo, 2013). Even though, according to Nurzadeh *et al.* (2021) and Bazarman *et al.* (2020), the

promising literature regarding miRNA-based therapy still has some unsolved challenges regarding miRNA delivery such as RNA instability, poor integration into the genome, innate immune response or low cellular uptake among others. Nevertheless, more in-depth knowledge is required to develop these strategies and provide a novel approach into personalized breast cancer treatment (Kaboli *et al.*, 2015).

Melatonin

Melatonin is a small indolamine secreted by the pineal gland during the night-time with a broad spectrum of functions (Gaspar *et al.*, 2019). It derives from serotonin and can be synthesized in other organs apart from the pineal gland, acting as a intracellular mediator or paracrine signal (González-González *et al.*, 2019). Many of its actions are mediated by G-protein coupled melatonin receptors in cellular membranes; other actions seem to involve orphan receptors or binding to molecules such as calmodulin. This reversible, calcium-dependent binding allows the pineal hormone to modulate intracellular functions, as preventing ERα transcription and diminishing estradiol-ER affinity (Gaspar *et al.*, 2019; González, 2019). Additionally, melatonin can detoxify free radicals and related oxygen derivatives via receptor-independent pathways (Reiter *et al.*, 2010).

In general terms, melatonin actions can be summarized in: circadian rhythm synchronization (1), free-radical neutralizer, acting as an antioxidant hormone (2), immune response stimulation through cytokines and interleukin production in lymphocytes and monocytes (3) and anti-neoplastic function by exerting anti-estrogenic actions acting over hypothalamus-hypophysis-gonads axis, acting as a SERM or SEEM modulator (4) (Martínez-Campa *et al.*, 2017).

1. Mechanisms of cancer inhibition by melatonin

Melatonin's anti-tumoral action has been explored in both *in vivo* and *in vitro* models of carcinogenesis and it has been demonstrated in a wide variety of endogenous and environmentally induced cancers. As shown in **Figure 4** several mechanisms have been described for the well-known anti-tumoral effects of melatonin.

It is reported that melatonin exerts anti-proliferative actions through a G_1-S transition delay, thus increasing the ratio of cells in G_0/G_1 phase with a consequent reduction of cells in S phase, reducing DNA synthesis and lengthening cell cycle duration. This G_0/G_1 arrest is explained by an upregulation of p53 and p21 and a downregulation of cyclin D1. However, some literature reported low to none-melatonin effects in apoptotic processes (Alonso-González *et al.*, 2017).

Moreover, tumour growth is a balance between cell proliferation and cell death. Melatonin promotes apoptosis in different tumoral cells lines including MCF-7 cells by regulating some factors related with apoptosis such as p53, p21, Bax or Bcl-2. Telomerase activity, as explained before, is a key player in cell viability during cancerous processes. Scientific evidences reported that ER+ breast tumours had impaired telomerase activity when melatonin was present, meaning anti-telomerase activity of melatonin is closely related to its anti-estrogenic activity (Alonso-González *et al.*, 2017).

Angiogenesis is mainly driven by VEGF, promoting tumour expansion and growth. Melatonin can counteract this effect by negatively targeting VEGF, disrupting tube formation and tubular network assembly. This hormone can also inhibit some other factors related to tumour growth and neutralize reactive oxygen species, which in hypoxic situations stabilize HIF-α, both involved in neoangiogenesis (Alonso-González *et al.*, 2017; Martínez-Campa *et al.*, 2017).

Tumour invasiveness and motility levels highly influence cell metastatic potential. Cell surface adhesion molecules as E-cadherin and β-integrin are regulated by melatonin, as well as metalloproteinases 2 and 9, involved in basal membrane degradation. This modulates structural features towards a non-invasive phenotype. Melatonin was also related with stress fibbers and adhesion plaques formation, associated with cell attachment (Alonso-González *et al.*, 2017; Martínez-Campa *et al.*, 2017).

As mentioned before, melatonin stimulates immune response through cytokine and interleukin production in lymphocytes and monocytes. Moreover, it stimulates the recruitment of natural killer cells, monocytes, macrophages and lymphocytes, polarizing tumoral cells towards a favourable immunological environment. Additionally, stimulation of IL-2 production is the selected therapy for treating cancer processes. The pineal hormone was proved to enhance this interleukin production by modulating IL-2 receptor system. Thus, melatonin supplementation alone or in combination with IL-2 and in conjunction

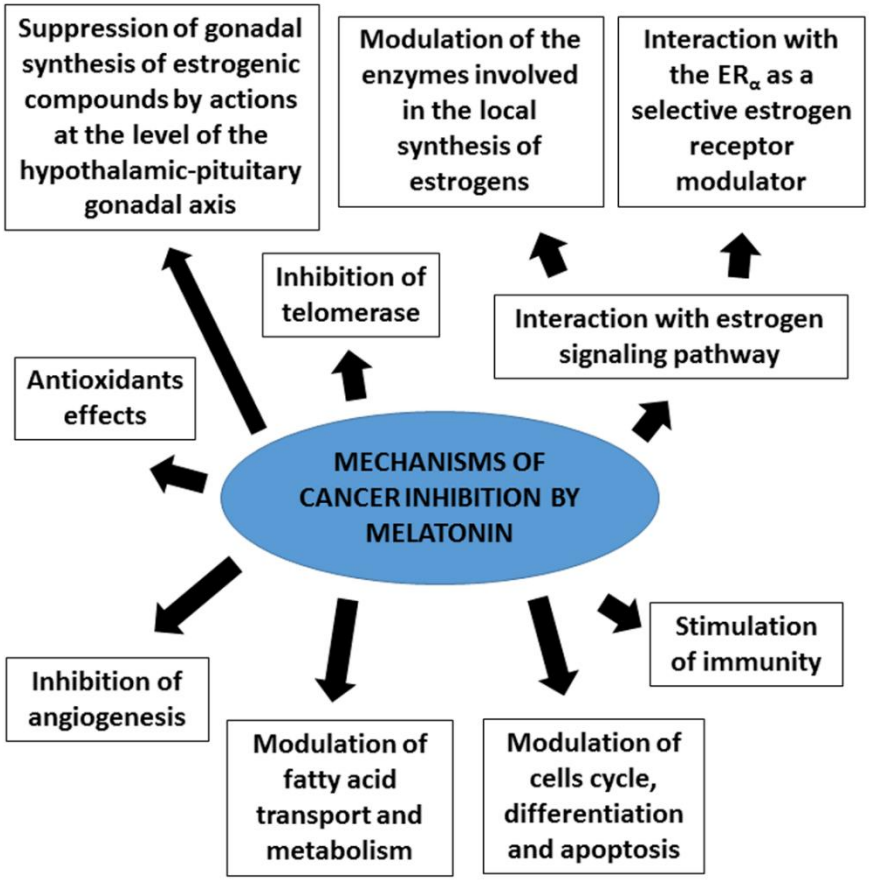


Figure 4. ***Anti-tumoral actions of melatonin in breast cancer.*** *Melatonin secretion exerts different receptor dependent or independent actions, related with breast cancer inhibition. Source: Alonso-González* et al. *(2020).*

with chemotherapeutic drugs significantly improves tumour regression and patient survival (Kubatka *et al.*, 2018).

Finally, epigenetic changes are considered as an important molecular alteration involved in cancer growth. Melatonin can also change DNA methylation patterns to reduce tumoral growth, acting as an epigenetic factor regulator (Alonso-González *et al.*, 2017).

2. Melatonin and breast cancer: anti-estrogenic actions

Melatonin plays an important role on hormone-dependent breast cancer inhibition due to its ability to interact with either estrogen synthesis (SEEM) or with the estrogen signalling pathway (SERM), counteracting the effects of estrogens. The mechanism involved in the antiestrogenic actions of melatonin includes a decrease on ERα expression and impairment of the E_2-ERα transcriptional activation. This effect seems to be mediated by calmodulin, which binds specifically to ERα but not to ERβ, acting as a calmodulin antagonist. Unlike other SERMs (tamoxifen or its derivates), melatonin does not bind to the ER nor changes its affinity that may interfere with estrogen binding to its receptor (Alonso-González *et al.*, 2017).

Estrogens increase cAMP in human breast cancer cells. Melatonin, through its binding to MT_1 membrane receptors, decreases cAMP, thus counteracting the estrogen-induced ERα transcriptional activity by interacting with the cAMP signaling cascade (Kiefer *et al.*, 2002).

As stated before, estrogens can be synthesized from sources different from the ovary. In post-menopausal women estrogens are synthesized in the mammary tissue by transformation either from androgen precursors or from biologically inactive estrogens. It has been widely demonstrated the ability of this indolamine to inhibit both the expression and activity the enzymes involved in the synthesis or transformation of biologically active estrogens from androgens. The anti-aromatase effects of melatonin depend on its binding to MT_1 receptors and have been demonstrated not only *in vitro* but also *in* vivo (Martínez-Campa *et al.*, 2017; Alonso-González *et al.*, 2017).

3. Melatonin as a sensitizing agent for chemotherapy

In the last decades, the effect of melatonin as an adjuvant agent has been investigated in many types of tumours using *in vitro* and *in vivo* models. Circadian disruption of nocturnal melatonin production by exposure to light at night has been associated with resistance to breast cancer therapy, including chemotherapy, suggesting that melatonin inhibition could play a role on resistance induced by chemotherapeutic drugs (Alonso-González *et al.*, 2017).

The underlying molecular mechanisms triggered by melatonin to overcome resistance are not clearly understood. For instance, several studies in different cell tumour lines demonstrated the ability of melatonin to decrease the expression of proteins involved in the drugs efflux from the cells, thus increasing cytotoxic drug effects (Alonso-González *et al.*, 2020).

Apoptosis resistance is another mechanism of resistance to chemotherapy in breast cancer cells. It has been postulated that melatonin, through the downregulation of COX-2 and PI3K/AKT signalling pathway among others, is able to potentiate apoptosis in cancer cells treated with different chemotherapeutic drugs (Alonso-González *et al.*, 2017).

In breast tumour fibroblasts, melatonin potentiates docetaxel and vinorelbine effects on differentiation and aromatase activity by decreasing cyclooxygenases expression, which makes it a promising adjuvant for chemotherapy sensibilization. Besides *in vivo* and *in vitro* experiments, several clinical trials have analysed the effects of melatonin as a chemotherapy adjuvant in different types of cancer including breast cancer, demonstrating that this indolamine protects against undesirable side effects, increases tumour response to treatment and also 1-year survival rate (Alonso-González *et al.*, 2020).

4. Melatonin as a sensitizing agent for radiotherapy

In recent years, several studies have showed that melatonin administration combined with radiotherapy is able to enhance its therapeutic effects and protect normal cells against side effects of this treatment, acting as a radiosensitizer. These radiosensitizing effects of this indolamine has been demonstrated both *in vivo* and *in vitro* and include mechanisms such as DNA repair impairment in tumour cells, leading to apoptosis and cell death triggered by p53 upregulation; modulation of inflammatory response reducing ROS production and DNA damage in normal tissues. It was also seen that melatonin can increase oxidative stress at tumoral level and modulate estrogen biosynthesis, leading to lower proliferation rates in breast cancer cells subjected to ionizing radiation, apart from modulating angiogenesis through inhibition of pro-angiogenic factors (Alonso-González *et al.*, 2020).

Within the tumour microenvironment, melatonin may also modulate pre-adipocytes response to ionizing radiation (differentiation, aromatase activity and expression). Furthermore, the inhibition of glycolysis by melatonin in tumour cells also increases radiation response. In summary, all these findings position melatonin a potential adjuvant molecule for breast cancer treatment. Mainly because of its protective and sensitizing effects, increasing cellular response when administered in combination with conventional breast cancer treatments (Alonso-González *et al.*, 2020).

OBJECTIVES

Melatonin is a pineal hormone with oncostatic properties that reduce the growth and development of hormone-dependent tumours, breast cancer included. More recently, melatonin has been considered as an adjuvant to chemotherapy or radiation, due to its ability to enhance the efficacy of these treatments. Therefore, the main objective of this work is to deepen into the molecular mechanisms involved and characterize the intracellular signaling pathways related to the sensitizing effects of melatonin to chemotherapy and radiotherapy using ER+ MCF-7 breast cancer cells as a model. In this regard, with this project we aim to complete the following specific objectives:

- To study if melatonin is able to potentiate the anti-proliferative effects of radiation and chemotherapeutic agents (docetaxel and doxorubicin).
- To analyse the changes induced by chemotherapy and ionizing radiation in the pattern of miRNA expression and to address the ability of melatonin to modulate those changes.
- To characterize the changes induced by radiation in the phosphoproteome and to evaluate the role of melatonin in the modulation of these molecular mechanisms.

MATERIALS AND METHODS

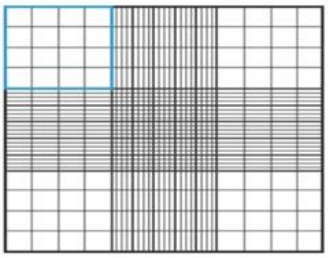
Cell culture

General conditions

Cell culture experiments were performed using MCF-7 cells, a non-metastatic and triple-positive breast tumour cell line purchased from the American Tissue Culture Collection (ATTC nº HTC-22™) (Rockville, MD, USA). These cells were cultured as monolayer in 75 cm^2 plastic flasks in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (20 units/ml) and streptomycin (20 µg/ml) (all from Lonza, Switzerland) and incubated at 37 ºC with 5% CO_2 to maintain a humid atmosphere.

Cell number determination

The total number of cells and their viability was determined using a Neubauer cell counting chamber. The full grid on the hemocytometer contains nine squares framed within an area of 1 mm^2 (**Figure 1**). The central counting area of the hemocytometer contains 9 large squares with 16 smaller squares inside. For cell counting, the chamber is previously mounted by placing a coverslip on top of the slide, allowing the suspension to flow due to capillarity. After this, 20 µL of cell suspension were dispensed into the chamber. Cells were counted using a fluorescence microscope (NIKON TMS, Germany), considering cells situated inside the central and four corner squares. The formula showed in **Figure 1** was used to calculate the number of cells present in the cell suspension.



$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

***Figure 1.* Hemocytometer gridlines.** *Hemocytometer diagram indicates one of the sets of 16 squares used for counting. (#) indicates the number of squares counted.*

Reagents and treatments

Two different chemotherapeutic drugs were used in the experiments carried out in this project: docetaxel and doxorubicin. On one hand, docetaxel (Sigma-Aldrich), is a taxane that binds and stabilizes the β−tubulin subunit of microtubules, preventing depolymerization of the mitotic spindle. On the other hand, doxorubicin (Sigma-Aldrich), is a cytotoxic anthracycline which intercalates within DNA base pairs, causing inhibition of both DNA and RNA synthesis. Both drugs were diluted in ethanol at a final

concentration of 0.1 M (stock) and stored at -20 ºC. The final concentration in culture media was either 1 µM or 1 nM, being the final concentration of ethanol lower than 0.0001%.

Melatonin stock (Sigma-Aldrich) was diluted in ethanol as well, achieving a final concentration of 100 mM, stored at -20 ºC. Melatonin used in culture media had a final concentration of 1 nM, a dose considered equivalent to the physiological concentration during night-time.

Ionizing radiation

MCF-7 cells were exposed to X-ray irradiation using YXLON SMART 200 tube (Yxlon International, Germany) at the Department of Radiology and Medical Physics of the University of Cantabria. Radiation was administered as an only-dose in a 11.5 cm x 8.5 cm field size. The source-half-depth distance was initially calculated to obtain a constant dose rate of 0.92 Gy/min. The radiation dose used was 8 Gy as previously described in our laboratory (Alonso-González, 2015).

Cell Proliferation assay

MCF-7 cells at 70-80% of confluency were initially cultured for 24 hours in DMEM supplemented with 0.5% dextran-charcoal stripped FBS (csFBS) and incubated at 37 ºC for 24 hours to allow cellular attachment. Melatonin pre-treated cells were incubated for 7 days in DMEM supplemented with 10% FBS containing 1 nM melatonin before being seeded into 96-multiwell plates. Cells were seeded at a density of 8 x 10^3 cells per well and 24 h later, melatonin pre-treated and control MCF-7 culture plates were irradiated at a dose of 8 Gy. Control cells were removed from the incubator and placed for the same period of time into the irradiator but without receiving any radiation.

Regarding chemotherapy studies, the procedure was the same as the described above. When cells were treated with chemotherapeutic agents, docetaxel and doxorubicin, both compounds were diluted in the culture media to achieve a final concentration of 1 nM.

In all cases, cells were incubated for 6 days and after that time, cell proliferation was measured by the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method. MTT (Sigma-Aldrich) is a yellow reagent that is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active. Therefore, conversion can be directly related to the number of viable cells. MTT was dissolved in Phosphate Buffer Saline (PBS) at a dose of 1 mg/mL and added 100 µL of the mixture to each well. It is important to conduct this step avoiding light exposure. Plates were incubated at 37 ºC for 4 h to allow formation of formazan crystals. Then, crystals were dissolved by adding a solution of 1 g of Sodium Dodecyl Sulphate (SDS, Pancreac AppliChem, USA) in 10 mL of HCl. After a 24 h incubation period, cell proliferation was determined by measuring absorbance levels at 570 nm using a microplate reader spectrophotometer (*Labsystems MultiSkan* RC

351, USA), assuming that an increase in cell number is directly related to the increase in absorbance due to the amount of MTT formazan formed.

Total RNA extraction

As previously described, MCF-7 cell cultures were pre-treated with melatonin 1 nM for 1 week before seeding. Later, cells were seeded into 6-well plates at a density of 1 x 10^6 cells per well in DMEM supplemented with 10% FBS. The following day, plates were either irradiated at 8 Gy or treated with chemotherapeutic agents (docetaxel or doxorubicin) at a dose of 1 µM. After a 4 h incubation step, total cellular RNA, including miRNA and other small RNA molecules, was isolated using the miRNeasy RNA kit (Qiagen, USA) following the manufacturer's instructions (**Figure 2**).

First, 700 µL of QIAzol lysis reagent were added to each well to homogenize the samples, using scrapers to scratch the well's base. Then, the homogenate was incubated at room temperature for 5 min. After chloroform addition, the homogenate was separated into three phases by a centrifugation step at 12000 g. The upper aqueous phase was transferred to a new collection tube containing 1.5 volumes of ethanol 100%, thus providing optimal conditions to bind RNA molecules to the affinity column. The mixture is then added to the RNeasy spin column and centrifuged at 9000 g at room temperature for 15 s, providing that RNA binds to the membrane. After washing with buffer RPE to eliminate other contaminants, RNA was eluted by adding 40 µL of RNase-free water into the column. The quality and quantity of the RNA eluted were measured with a spectrophotometer (Nanodrop 1000 V 3.6). The ratio A_{260}/A_{280} was used to asses RNA purity, considering samples with a ratio between 1.9-2.0 as pure RNA.

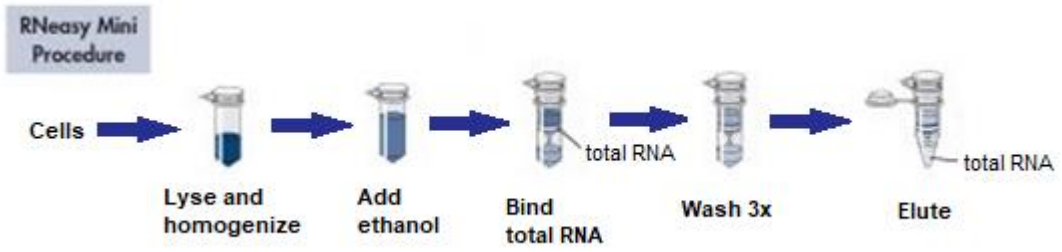


Figure 2. ***Total RNA extraction procedure (including miRNA) using miRNeasy RNA kit (Qiagen, USA).***

Conversion of mature miRNA into cDNA

In order to perform miRNA expression analysis, RNA samples were polyadenylated by poly(A) polymerase and subsequently converted into cDNA by a reverse transcription reaction using miScript II RT Kit (Qiagen, USA) (**Figure 3**). Both steps were performed in parallel in the same tube, using and oligo-dT primer with universal tag sequence on the 5' end, allowing amplification of mature miRNA in the qPCR analysis. For cDNA synthesis, 250 ng of total RNA were mixed with the different components according to the manufacturer's instructions (shown in **Table 1**). Each reaction was incubated in a thermocycler (MyCycler Gradient, Bio-Rad, USA). The protocol followed a two-step reaction: 60 min at

37 ºC and 5 min at 95 ºC to inactivate reverse transcriptase activity. Finally, each sample was diluted in 200 µL of RNase-free water and stored at -20 ºC until use.

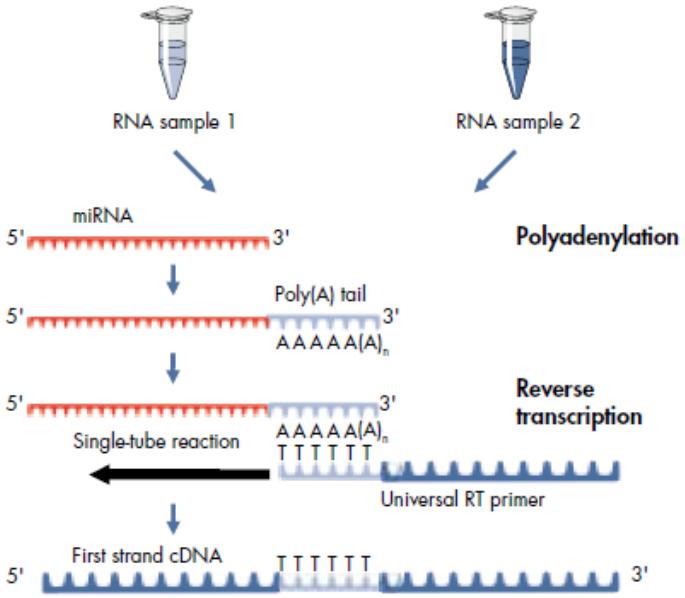


Figure 3. ***cDNA synthesis procedure by using miScript RT II kit (Qiagen, USA).***

Table 1. ***cDNA synthesis reagents and correspondent volumes.*** *Volumes are expressed in (µL).*

Component	Volume
miScript HiSpec Buffer	4
miScript Nucleics Mix	2
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2
Template RNA	Variable
Total volume	20

Breast Cancer Pathway-Focused miRNA PCR array

To study changes in miRNAs expression through different treatments, a pathway focused miRNA expression profiling was performed using a Human Breast Cancer microarray (MIHS-109ZA, Qiagen, USA). Specifically, this array consists of 84 mature miRNA forward primers arrayed in miRNome panels, that appear to be somehow related to breast cancer. Each well contains the components required to ensure that each quantitative PCR reaction (qPCR) will generate a single, gene-specific amplicon, preventing the co-amplification of non-specific products. The array also contains miScript PCR controls that enable normalization: an array normalizer (*C. elegans* miR-39), six normalization controls (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6B/RNU6-2), a reverse transcription control (miRTC) and a positive PCR control (PPC), as shown in **Figure 4**.

1 hsa-let-7a-5p	9 hsa-miR-1-3p	17 hsa-miR-129-5p	25 hsa-miR-152-3p	33 hsa-miR-181c-5p	59 hsa-miR-222-3p
2 hsa-let-7b-5p	10 hsa-miR-100-5p	18 hsa-miR-130a-3p	26 hsa-miR-155-5p	34 hsa-miR-181d-5p	60 hsa-miR-223-3p
3 hsa-let-7c-5p	11 hsa-miR-107	19 hsa-miR-130b-3p	27 hsa-miR-15a-5p	35 hsa-miR-182-5p	61 hsa-miR-25-3p
4 hsa-let-7d-5p	12 hsa-miR-10a-5p	20 hsa-miR-132-3p	28 hsa-miR-15b-5p	36 hsa-miR-186-5p	62 hsa-miR-26a-5p
5 hsa-let-7e-5p	13 hsa-miR-10b-5p	21 hsa-miR-140-5p	29 hsa-miR-16-5p	37 hsa-miR-18a-5p	63 hsa-miR-26b-5p
6 hsa-let-7f-5p	14 hsa-miR-125b-5p	22 hsa-miR-141-3p	30 hsa-miR-17-5p	38 hsa-miR-193b-3p	64 hsa-miR-27a-3p
7 hsa-let-7g-5p	15 hsa-miR-125b-1-3p	23 hsa-miR-145-5p	31 hsa-miR-181a-5p	39 hsa-miR-195-5p	65 hsa-miR-27b-3p
8 hsa-let-7i-5p	16 hsa-miR-128-3p	24 hsa-miR-148a-3p	32 hsa-miR-181b-5p	40 hsa-miR-199b-3p	66 hsa-miR-29a-3p
				41 hsa-miR-199a-5p	67 hsa-miR-29b-3p
				42 hsa-miR-19a-3p	68 hsa-miR-29c-3p
				43 hsa-miR-19b-3p	69 hsa-miR-31-5p
				44 hsa-miR-200a-3p	70 hsa-miR-328-3p
				45 hsa-miR-200b-3p	71 hsa-miR-340-5p
				46 hsa-miR-200c-3p	72 hsa-miR-424-5p
				47 hsa-miR-202-3p	73 hsa-miR-429
				48 hsa-miR-203a-3p	74 hsa-miR-485-5p
				49 hsa-miR-204-5p	75 hsa-miR-489-3p
				50 hsa-miR-205-5p	76 hsa-miR-495-3p
				51 hsa-miR-206	77 hsa-miR-497-5p
				52 hsa-miR-20a-5p	78 hsa-miR-548c-3p
				53 hsa-miR-20b-5p	79 hsa-miR-607
				54 hsa-miR-21-5p	80 hsa-miR-613
				55 hsa-miR-210-3p	81 hsa-miR-7-5p
				56 hsa-miR-212-3p	82 hsa-miR-93-5p
				57 hsa-miR-214-3p	83 hsa-miR-96-5p
				58 hsa-miR-22-3p	84 hsa-miR-98-5p

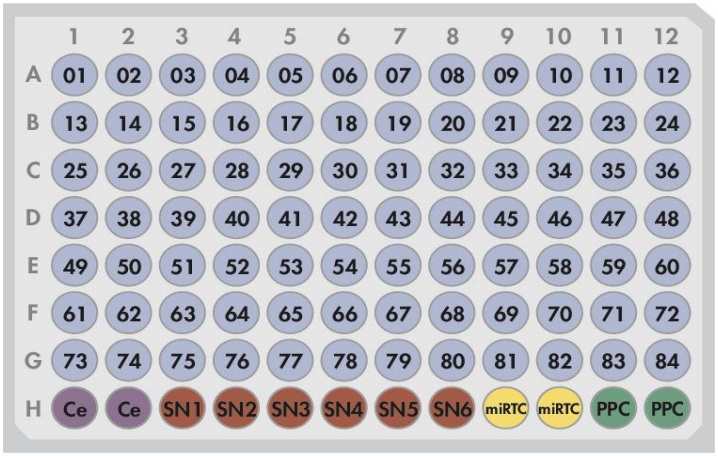


Figure 4. ***MIHS-109ZA Human Breast Cancer microarray scheme for 96-well plates.*** *Wells A1 to G12 contain selected miRNAs related to breast cancer (listed on the top-right tables). Wells H3 to H8 each contain an assay for a different snoRNA/snRNA that can be used as a normalization control for the array data. Wells H1 and H2 contair replicate* C. elegans *miR-39 can be used as an alternative normalizer for array data (Ce). Wells H9 and H10 contain replicate reverse transcription controls (miRTC). Wells H11 and H12 contain replicate positive PCR controls (PPC).*

Real-time PCR for miRNA PCR Array

The PCR array performs miRNA expression analysis with qPCR sensitivity and the multi-gene profiling capability of a microarray. The cDNA obtained from RNA extraction samples was mixed with the reagents provided by the miScript SYBR Green PCR Kit (Qiagen, USA), as shown in **Table 2**. This Master mix contains a miScript universal primer as a reverse primer that allows the detection of mature miRNAs in combination with miScript specific primers attached to the array plate wells (forward primer) (**Figure 5**). For the 96-well plate, 25 µL of master mix were dispensed to each well. To avoid the presence of bubbles, plates were centrifuged at 1000 g at room temperature for 1 min. Afterwards, plates were placed in a qPCR thermocycler (CFX96 Bio-Rad, USA) using the following protocol: an initial activation step of 15 min 95 ºC with a ramp rate of 1º C/s and a three-step cycling based on a denaturation step (15 s; 94 ºC), an annealing step (30 s; 55 ºC) and an extension step (30 s; 70 ºC). Cycling step was repeated 40 times. Melting curves were plotted after the final cycling step to verify unspecific amplification of primers.

Table 2. ***PCR synthesis reagents and correspondent volumes.*** *Volumes are expressed in (µL) and calculated for the total of a 96-well plate, adding 25 µL per well.*

Component	Volume
QuantiTect SYBR Green PCR Master Mix	1375
miScript Universal Primer	275
RNase-free water	1000
Template cDNA	100
Total volume	2750

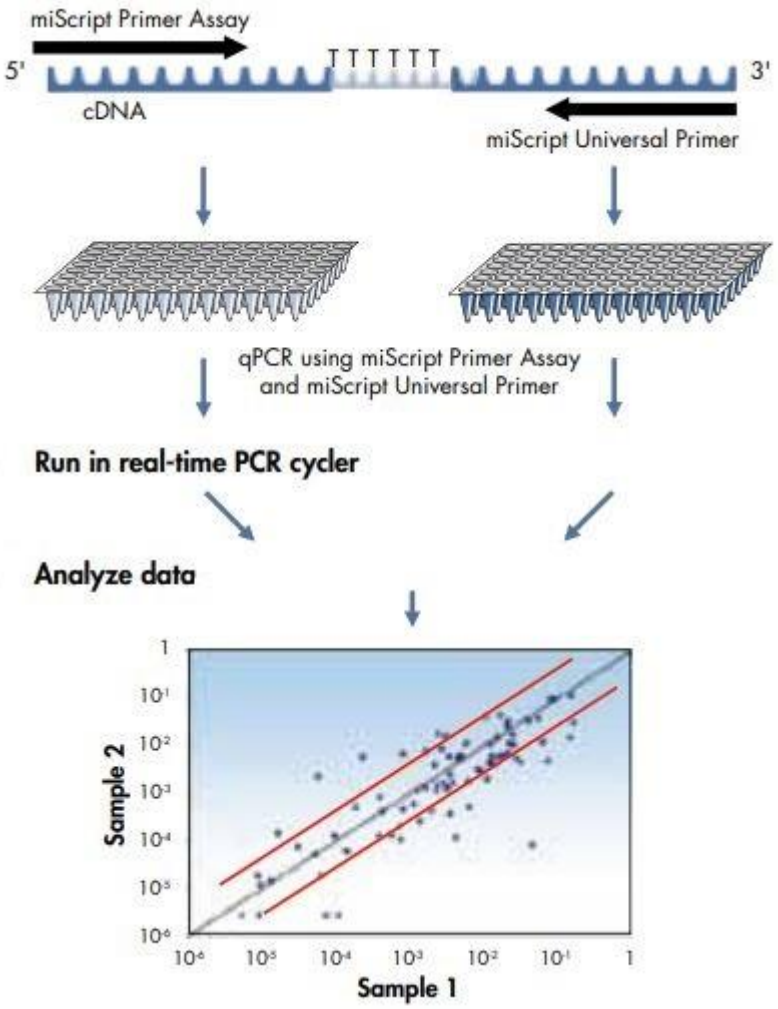


Figure 5. ***miScript PCR microarray procedure by using MIHS-109ZA Human Breast Cancer microarray (Qiagen, USA).***

Data obtained by qPCR were analyzed using Qiagen GeneGlobe online tool for microarray data management. C_T values were exported from the original qPCR software and transcribed into GeneGlobe Analyze section, obtaining fold change data (based on $\Delta\Delta C_T$ method), sample quality assessments and different plots. This information was used for miRNA selection between different experimental groups to perform specific qPCRs.

Analysis of specific miRNAs by qPCR

Selected miRNAs from the PCR microarray were subjected to specific qPCR to further validate their expression profiles. RNA extraction samples were used to obtain cDNA by a reverse transcriptase reaction. Complementary DNA samples were mixed with the reagents provided by the miScript Primer Assays Kit (Qiagen, USA), as shown in **Table 3**. RNase-free water was added to the mix in order to complete the total volume of 25 µL/per well. SNORD68 and SNORD95 were used as normalization controls. Specific miRNAs selected were: miR-20a, miR-20b, miR-17, miR-141, miR-15a, miR-19a, miR-29a and miR-93. To avoid bubbles during qPCR plate lecture, plates were centrifuged at 1000 g at room temperature for 1 min. Afterwards, plates were placed in a PCR thermocycler (CFX96 Bio-Rad, USA) using the following protocol: an initial activation step of 15 min 95 ºC and a three-step cycling based on a denaturation step (15 s; 94 ºC), an annealing step (30 s; 55 ºC) and an extension step (30 s; 70 ºC), repeating the cycling step 40 times. Melting curves were plotted after the extension step of each cycle to verify unspecific amplification of primers.

Table 3. ***PCR synthesis reagents and correspondent volumes.*** *Volumes are expressed in (µL).*

Component	Volume
QuantiTect SYBR Green PCR Master Mix	12.5
miScript Universal Primer	2.5
miScript Specific Primer	2.5
RNase-free water	5
Template cDNA	2.5
Total volume	25

qPCR Data analysis

The $\Delta\Delta C_T$ method is the recommended data analysis tool for qPCR experiments. C_T values obtained for the normalization miRNAs primers (HKG) and miRNAs of interest (GOI) were determined in each sample. It is important to note that only C_T values less than 35 were used for the analysis. For each set of samples, the difference in ΔC_T values ($\Delta\Delta C_T$) for the miRNAs of interest between the samples was calculated. Specifically, the difference between the C_T values (ΔC_T) for each miRNA of interest and the average C_T value of the set of housekeeping miRNAs were calculated as follows:

$$\Delta C_T \text{ (control)} = C_T \text{ (GOI)} - C_T \text{ (HKG)}$$

$$\Delta C_T \text{ (experimental)} = C_T \text{ (GOI)} - C_T \text{ (HKG)}$$

The difference in ΔC_T values ($\Delta\Delta C_T$) between the two samples were calculated as:

$$\Delta\Delta C_T = \Delta C_T \text{ (experimental)} - \Delta C_T \text{ (control)}$$

Finally, the fold-change for each gene between the control group and the different treatments was calculated as $2^{(-\Delta\Delta CT)}$.

Phosphokinase screening

To determine the sensitizing effects of melatonin in radiation-induced changes in multiple kinases and their protein substrates we used a membrane-based sandwich immunoassay Human Phospho-Kinase

Array Proteome Profiler™ (R&D Systems, USA). This array allows the simultaneous detection of relative phosphorylation levels of 37 kinases. In particular, the membrane allows the antibodies to bind to specific target proteins present in our samples.

This study was performed on MCF-7 cells cultured in DMEM medium supplemented with 10% FBS and treated with radiation (8 Gy) either pre-treated or not with melatonin (1 nM). Cells were seeded into 6-well plates at a cellular density of 8 $x10^5$ cells per well. After a 4 h incubation, cells were washed twice with chilled PBS, lysed and mixed for 30 minutes in a rocking platform at 4 ºC. Cell lysates (containing 500 µg of protein) were then added to the previously blocked nitrocellulose membranes and incubated overnight at 2-8 °C on a rocking platform. After performing two PBS washes, antibody detection cocktails were added to the membranes and incubated for 2 h at room temperature. Once the incubation step was finished, streptavidin-HRP-conjugated secondary antibodies were used for chemiluminescent detection, generating a signal directly proportional to the amount of protein bound to the membrane. Finally, the membranes were exposed to a film using a chemical reagent mix with hydrogen peroxide and luminol. Pixel densities on the X-ray film were collected and analyzed using a LI-COR Odyssey IR Imaging System V3.0 (LI-COR Odyssey Biosciences, USA).

Analysis of protein levels

Protein extraction

Western Blot analysis was used to further validate the most relevant changes observed in the phosphokinase assay, which included Akt, p70S6 kinase and their active phosphorylated versions in residues Thr308 for p-Akt and Thr389 for p-p70S6. MCF-7 control and melatonin pre-treated cells were seeded and irradiated as described above, performing protein extraction after a 4 h incubation period. For that purpose, each well was washed twice with 1 mL of chilled PBS. Then cells were lysed by adding 0,5 mL of RIPA buffer per well, containing phosphatase inhibitors (1:100) and protease inhibitors (1:250). To facilitate protein lysis, the well's base was scratched using scrapers. Samples were placed in a 2 mL Eppendorf and stored at -20 ºC.

Protein concentration was assessed by Bradford colorimetric method in a spectrophotometer at 620 nm wavelength. Briefly, Bradford method is based on the bond between Coomassie Blue dye and the proteins of interest, specifically binding to the blue form of this dye. This protein-colorant complex has a higher molar extinction coefficient than free colorant, allowing protein detection. After this, a standard curve was generated using Bovine Serum Albumin (BSA, Sigma-Aldrich, USA). Hereinafter, samples were mixed at a 1:1 ratio with a mix of Laemmli and β-mercaptoethanol 5% (Bio-Rad, USA) using a vortex. Afterwards, the mix was heated at 98 ºC in a dry-bath for 10 min. A centrifugation step was performed at 14000 g at 4 ºC for 5 min. Supernatant was isolated from the samples and stored at -70 ºC until use.

Western blotting

To perform electrophoresis, 25 µg of each protein sample were loaded to a 10% SDS-PAGE gel (SDS-polyacrylamide), using 3 µL of NZYBlue (NZYtech, Portugal) molecular weight marker as a reference. SDS-PAGE gels were done prior to electrophoresis procedure, using the following components (**Table 4**):

Table 4. ***Electrophoresis gel components and volumes.*** *Volumes are expressed in mL. Resolving buffer was done by mixing 90.85 g of Tris in 20 mL of SDS 10 % adding HCl until reaching a pH of 8.8. Stacking buffer was done by mixing 30.3 g Tris in 20 mL of SDS 10 % adding HCl to adjust pH to 6.8.*

	Volume	
Component	**Resolving gel**	**Stacking gel**
Distilled water	5	1.562
Acrylamide/Bis-acrylamide 40 %	2.5	0.313
Resolving buffer (pH 8,8)	2.5	-
Stacking buffer (pH 6,8)	-	0.625
Ammonium persulfate (APS) 10 %	0.150	0.042
Temed	0.0075	0.0075

The gel was placed in an electrophoresis tank loaded with migration buffer 1X (Tris 25 mM pH 8.5; glycine 0.2 M; SDS 0.1%). A constant voltage of 100 V was applied to the tank for 15 min until the electrophoresis front reached the resolving gel. Afterwards, a constant voltage of 160 V was applied for 50 min.

After this, gels were removed from the gel cassette and immersed in transference buffer (Tris 25 mM pH 8.3; glycine 192 mM; methanol 20%) for 30 min in a rocking platform. Immediately after, the transference sandwich was assembled. In each cassette, gel and the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) are put in tight contact and surrounded by filter paper and fibber pads, all soaked in transference buffer. Transference cassettes were placed in a transference tank loaded with chilled transference buffer and subjected to a constant voltage of 100 V at 4 ºC for 100 min. Protein transference efficacy was assessed by membrane staining with Ponceau S red and gel staining with Coomassie Blue.

For membrane blockage, a solution of BSA 3% in TBS-T (Tris-HCl 10 mM, pH 7.6, NaCl 150 mM, Tween 20 0.05%) was added to the membranes, letting it sit for 1 h at room temperature. This avoids unspecific binding of primary antibodies. The following step was incubating the membranes with the selected primary antibody diluted in blocking solution at 4 ºC overnight in a rotary shaker. Afterwards, three washing steps of 10 min using TBS-T were performed in a rocking platform. Later on, membranes were incubated with a secondary antibody with a fluorescent dye diluted in blocking solution for 1 h at room temperature. After incubation, three washing steps, following the aforementioned conditions, were done. Selected antibodies description is shown in **Table 5**.

To correct differences while loading samples in the gel, membranes were incubated with actin antibody for 24 h at 4 ºC, serving as a normalizing control. Fluorescence signal was detected using LI-COR

Odyssey IR Imaging System V3.0 (LI-COR Odyssey Biosciences, USA). To study differences in optical density bands, we used Image Studio and ImageJ software. Three different protein extracts were analysed and data were represented as percentage of control (non-treated cells).

Table 5. ***Antibody specifications.***

	Antibody	Dilution	Source	Molecular weight (kDa)
Primary	Akt (pan) (Cell Signalling, USA)	1:250	Monoclonal/Rabbit	60
	Phospho-Akt (Thr 308) (Cell Signalling, USA)	1:250	Monoclonal/Rabbit	60
	p70S6 Kinase (Thr 389) (Cell Signalling, USA)	1:250	Monoclonal/Rabbit	70,85
	Phospho-p70S6 Kinase (Cell Signalling, USA)	1:250	Policlonal/Rabbit	70,85
	Actin	1:1000	Monoclonal/Mouse	42
Secondary	Anti-rabbit IRDye-680RD (LI-COR Odyssey Biosciences, USA)	1:10000	-	-
	Anti-mouse IRDye-800CW (LI-COR Odyssey Biosciences, USA)	1:10000	-	-

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM) from three independent experiments. Statistical differences between groups were processed by One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls test, with $p < 0.05$, $p < 0.01$ and $p < 0.001$ considered to be statistically significant.

Image Studio 5.2 and ImageJ software were used for protein analysis, CFX Maestro 2.0 and Qiagen GeneGlobe Analyze tool for qPCR data analysis and GraphPad Prism5 software for plotting data and statistics.

RESULTS

Radiation and melatonin

Effects of ionizing radiation and melatonin on MCF-7 cell proliferation

The first objective of this work was to corroborate the oncostatic properties of melatonin on MCF-7 cell proliferation, as well its ability to enhance the anti-proliferative effect of radiation. Previous results from our laboratory had established 8 Gy as the optimal radiation dose for MCF-7 cells. As shown in **Figure 1**, with radiation alone the inhibition of cell proliferation was 45% and 33% after 3 (**Figure 1A**) and 6 days (**Figure 1B**) of incubation after irradiation. Finally, the greatest inhibition on cell proliferation was found when cells were pre-treated with the indolamine before being radiated, showing a reduction of 58% after 3 days and 62% after 6 days when compared to non-radiated cells.

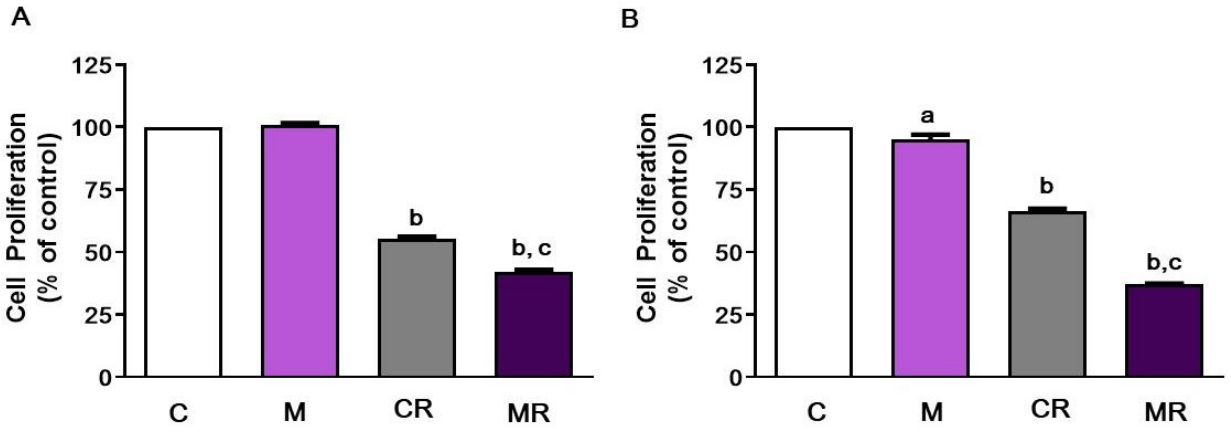


Figure 1. ***Effect of melatonin pre-treatment and ionizing radiation on MCF-7 cell proliferation.*** *Cells were pre-treated with melatonin 1 nM for one week, radiated at 8 Gy or a combination of both treatments. Cell proliferation was measured at 3* ***(A)*** *or 6* ***(B)*** *days after radiation. Data are expressed as percentage of control non-radiated cells (mean ± SEM). a, p<0.01 vs. C; b, p<0.001 vs. C; c, p<0.001 vs. CR.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***CR:*** *Radiated cells (8 Gy);* ***MR****: Melatonin pre-treated and radiated cells.*

Effects of ionizing radiation and melatonin on the expression of breast cancer-related miRNAs

To study the effects of melatonin and radiation on miRNA expression patterns in MCF-7 cells, we employed a Human Breast Cancer microarray (MIHS-109ZA, Qiagen, USA). This array contains 84 mature miRNA that appear to be somehow related to breast cancer. Establishing a fold-change of ± 2, melatonin pre-treatment showed a downregulation of 27 miRNAs and an upregulation of 6 miRNAs when compared to control cells (**Figure 2A**). In a similar way, radiation alone modified the expresion of 33 miRNAs (29 downregulated and 4 upregulated) as we can see in the heatmap showed in **Figure 2B**. Finally, the most significant results in fold-change analysis were observed in those cells that were pre-treated with melatonin for one week before being irradiated. In this case, 37 miRNAs were downregulated and 6 upregulated compared to non-radiated control cells (**Figure 2C**).

It is important to specify that the membranes included in the miRNA microarray contain only one well for each of the miRNAs analyzed. Therefore, the results showed in **Figure 2** may serve as a preliminar study of what may be happening with each of the treatments analyzed in this work. Thus, these results must be confirmed by specific qPCR. For this project, due to their relevance in breast cancer onset and progression, a set of 8 miRNAs (miR-20a, miR-20b, miR-17, miR-141, miR-15a, miR-19a, miR-29a and miR-93), were selected to be studied specifically. The relative quantity (ΔC_T) of these specific miRNAs is shown in **Figure 2D.**

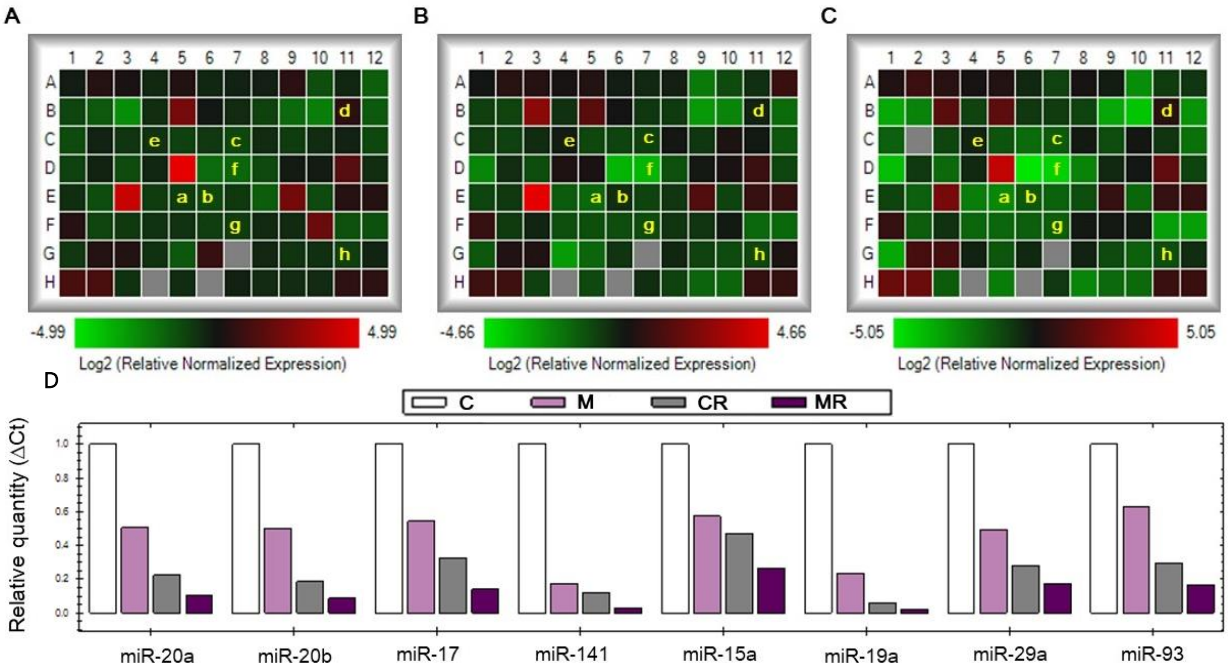


Figure 2. ***Effects of melatonin and radiation on breast cancer miRNAs expression microarray****. Cells were pre-treated with melatonin (1 nM) for one week and/or radiated (8 Gy). Total RNA was extracted after 4 h, reverse transcribed and used for qPCR analysis using Human Breast Cancer microarray (MIHS-109ZA). Panels* ***A-C*** *show heatmaps of relative normalized expression between different treatments* ***(A)*** *M vs C;* ***(B)*** *CR vs C;* ***(C)*** *MR vs C.* ***(D)*** *Bar chat of relative normalized expression (ΔC_T) of selected miRNAs. a: miR20a; b: miR-20b; c: miR-17; d: miR-141; e: miR-15a; f: miR-19a; g: miR29a; h: miR-93.* ***C:*** *Control;* ***M:*** *Melatonin pre-treated cells;* ***CR:*** *Radiated cells;* ***MR****: Melatonin pre-treated and radiated cells.*

Results of microarray miRNA profiles were further validated by specific qPCR. When MCF-7 cells were pre-treated with 1 nM of melatonin, the indolamine significantly decreased the expression levels of miRs 20a, 20b, 19a, 29a and 93 in comparison to control cells. Similar results were found when cells were irradiated. Surprisingly, radiation treatment resulted in a significant overexpression of miRs 17, 141 and 15a. Nevertheless, when cells were treated with melatonin prior to radiation, this hormone was able to counteract the stimulatory effect of radiation (**Figure 3**).

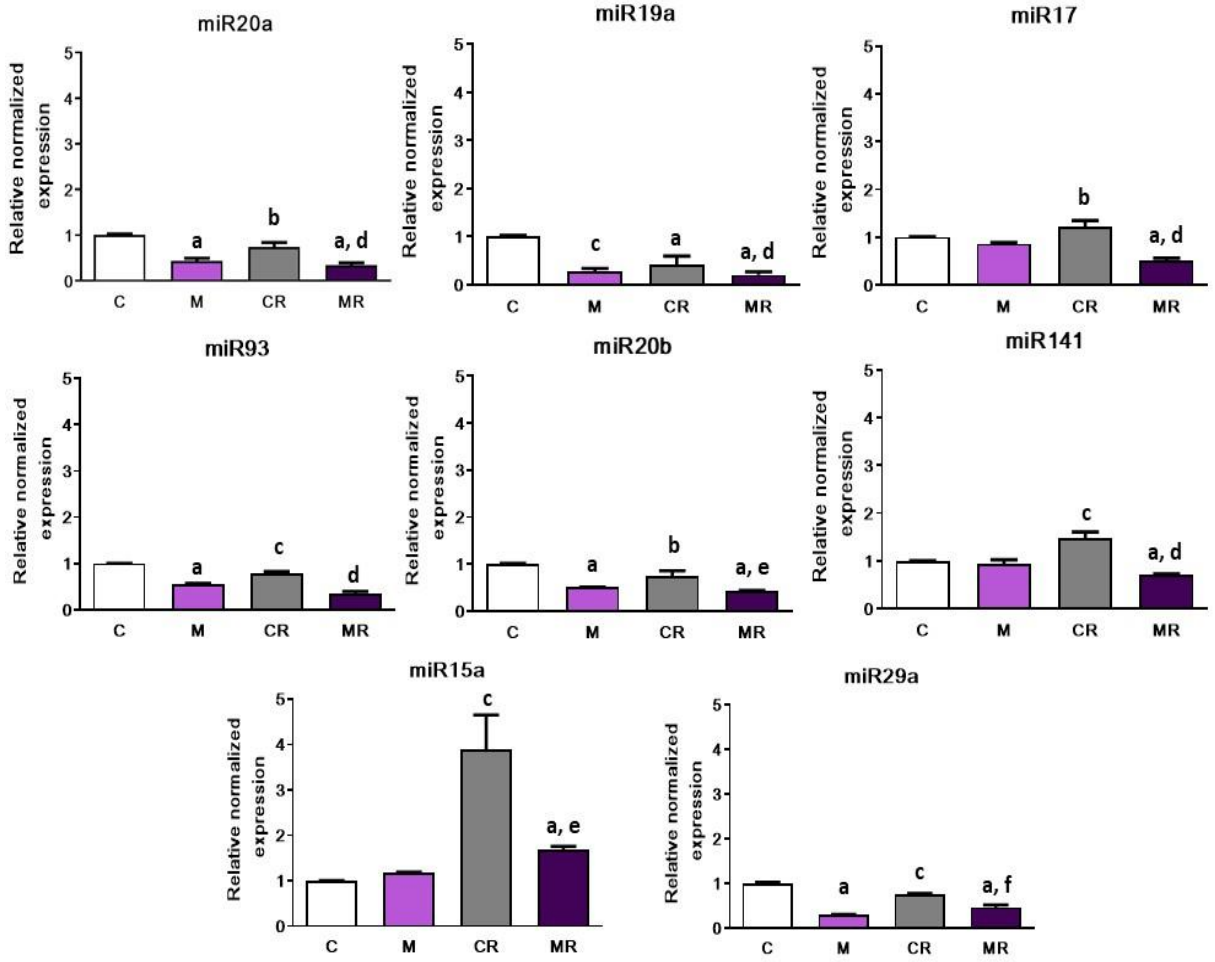


Figure 3. qPCR analysis of the specific miRNAs expression levels in MCF-7 cells. *Cells were treated with 1 nM melatonin and/or irradiated (8 Gy), and after 4 h total RNA was isolated and reverse transcribed. cDNA was subjected to qPCR using miRNA specific primers. Data are expressed as a relative normalized expression compared to control cells (mean ± SEM). a, p<0.001 vs. C; b, p<0.05 vs. C; c, p<0.01 vs. C; d, p<0.01 vs. CR; e, p<0.05 vs. CR; f, p<0.001 vs. CR.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***CR:*** *Radiated cells (8 Gy);* ***MR****: Melatonin pre-treated and radiated cells.*

Changes on kinase intracellular regulators induced by melatonin and radiation

To study the modulatory effects of melatonin in different signaling pathways related with cell proliferation and survival, we investigated the changes in the activation or inactivation status of different protein kinases, using the Proteome Profiler™ Array (R&D Systems, USA). As shown in **Figure 4A**, the most relevant results from the array panels were found in two proteins belonging to the mTOR signaling pathway: Akt (Thr308), which phosphorylates mTOR directly; and the ribosomal protein p70S6 kinase (Thr389), one of the downstream substrates of mTORC1, leading to cell survival and proliferation when activated. Radiation alone induced a reduction on the relative phosphorylation levels of both proteins compared to control cells. These inhibitory effects were also potentiated when cells were pre-treated with melatonin prior to radiation (**Figure 4B**).

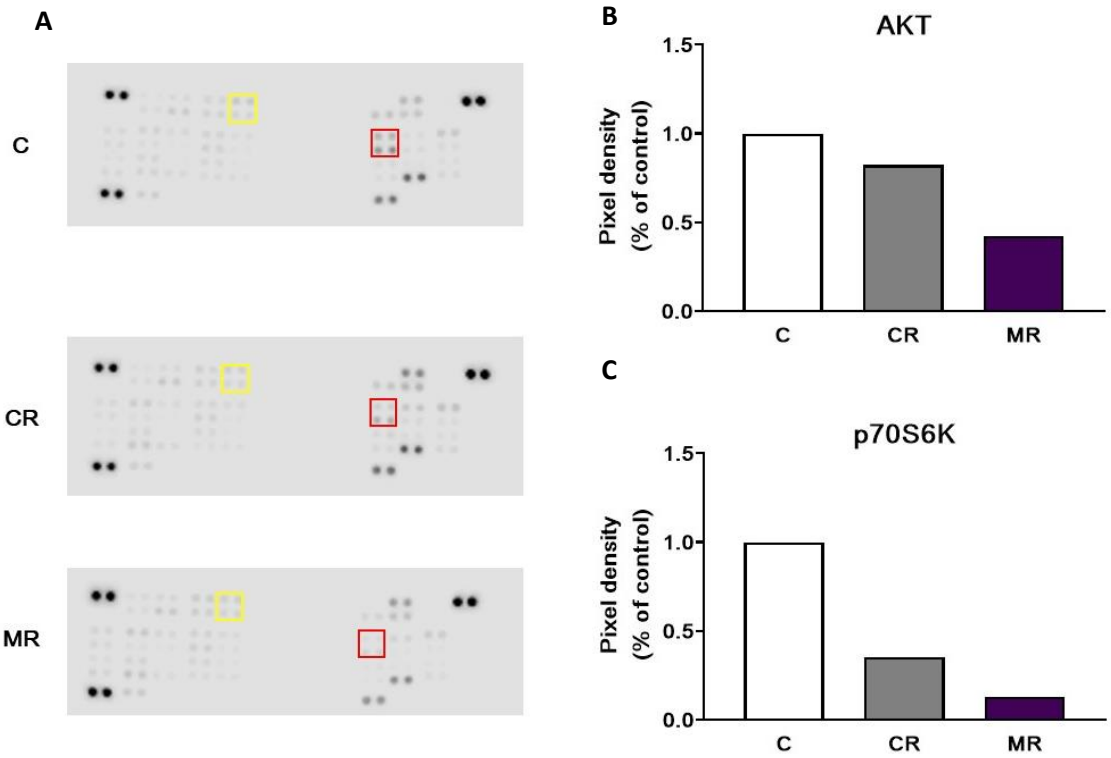


Figure 4. ***Effects of melatonin and radiation on the phosphorylation levels of Akt and p70S6 kinases. (A)*** *Representative images of Proteome Profiler array blots assessed with protein extracts from MCF-7cells pre-treated with melatonin and/or radiated (8 Gy). Dots corresponding to Akt levels are shown in yellow and p70S6K dots are shown in red.* ***(B)*** *Bar chart compiling quantification densitometry data of Akt dots, expressed as percentage of control.* ***(C)*** *Bar chart compiling quantification data of p70S6K, expressed as percentage of control.* ***C:*** *Control;* ***CR:*** *Radiated cells (8 Gy);* ***MR****: Melatonin pre-treated (1 nM) and radiated cells.*

To validate this data, we performed protein quantification studies in the different samples by western blot (**Figure 5**). In these experiments, a group of melatonin pre-treatment was also included to see specifical differences that were not determined in the phosphokinase assay. Surprisingly, radiation induced a significant increase in total Akt levels compared to control non-treated cells. In spite of this, melatonin pre-treatment was able to counteract this radiation effect (**Figure 5A**). In respect of Akt phosphorylated version (p-Akt), neither radiation nor melatonin modified its expression. Nevertheless, melatonin-pre-treatment prior to radiation showed a significant decrease in Akt activation compared to control cells (**Figure 5B**).

Regarding p70S6K, radiation slightly increased its total protein levels but once again this effect was counteracted by melatonin (**Figure 5C**). However, none of the treatments were able to induce significant changes in p70S6K phosphorylation (**Figure 5D**).

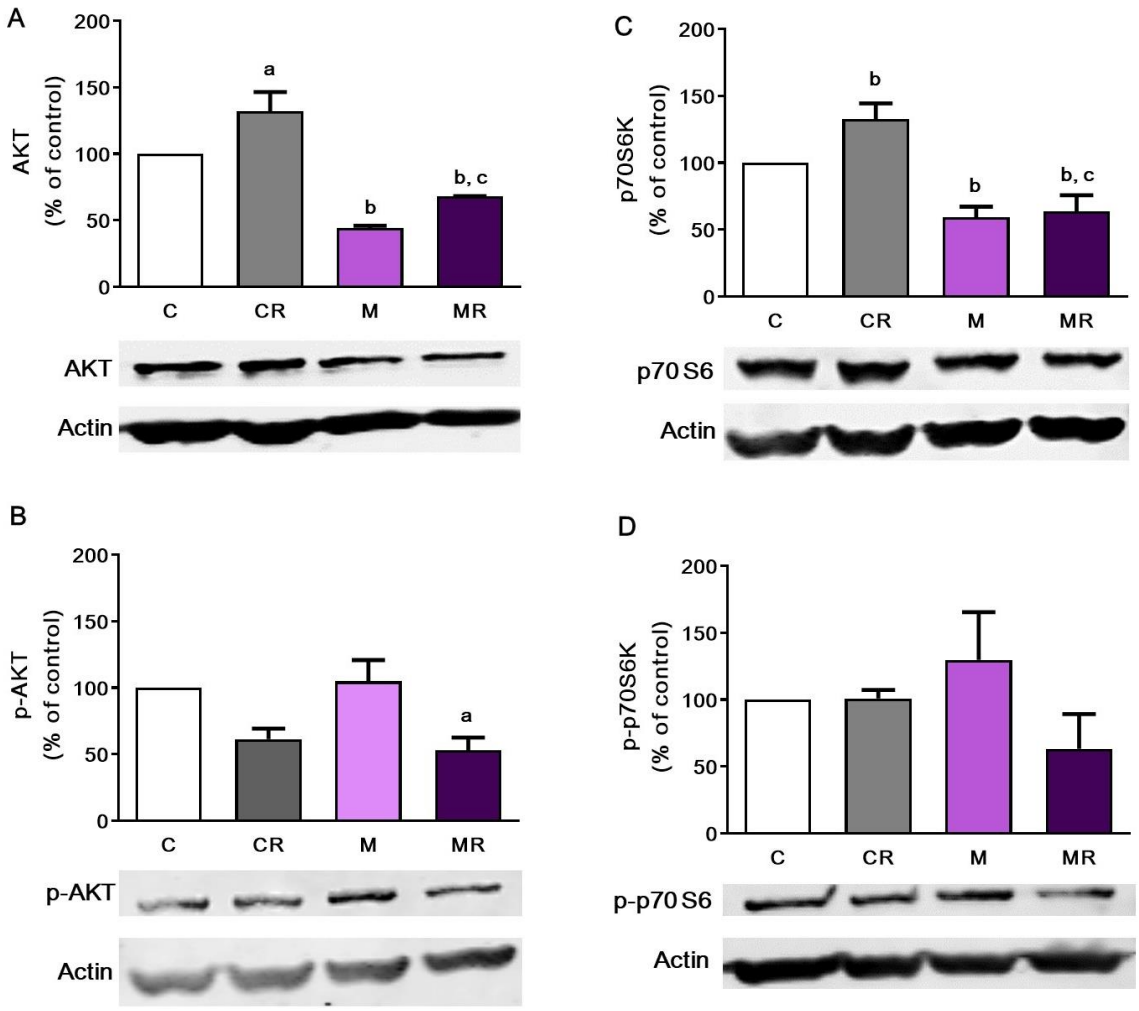


Figure 5. ***Effects of melatonin and radiation on total and phosphorylated Akt and p70S6 kinases.*** *Protein extracts from MCF-7 cells treated with 1 nM of melatonin, radiation (8Gy) or both were used for Western-Blot detection of total Akt* ***(A),*** *p-Akt (Thr308)* ***(B),*** *total p70S6K* ***(C)*** *and p-p70S6K (Thr389)* ***(D).*** *Bar chart data are expressed as % of control non-radiated cells, using β-actin as a loading control. a, p<0.05 vs. C; b, p<0.001 vs. C; c, p<0.001 vs. CR.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***CR:*** *Radiated cells (8 Gy);* ***MR****: Melatonin pre-treated and radiated cells.*

Chemotherapy and melatonin

Effects of chemotherapeutic agents and melatonin on MCF-7 cell proliferation

Another point of this work was to address whether melatonin could sensitize MCF-7 cells to chemotherapeutic agents (docetaxel and doxorubicin). To illustrate this, cell proliferation experiments were conducted, showed in **Figure 6** and **Figure 7**.

We first wanted to determine the effects of different docetaxel doses on MCF-7 cell proliferation. As expected, docetaxel inhibited cell proliferation in a dose dependent manner (**Figure 6A**) after 6 days of incubation. Based on these results, we selected sub-pharmacological doses of docetaxel (1 nM) to test the ability of melatonin to potentiates docetaxel effect. As we can see on **Figure 6B**, melatonin pre-treatment at physiological concentrations (1 nM) potentiates the docetaxel-induced growth inhibition, leading to an inhibition of 75% compared to control cells.

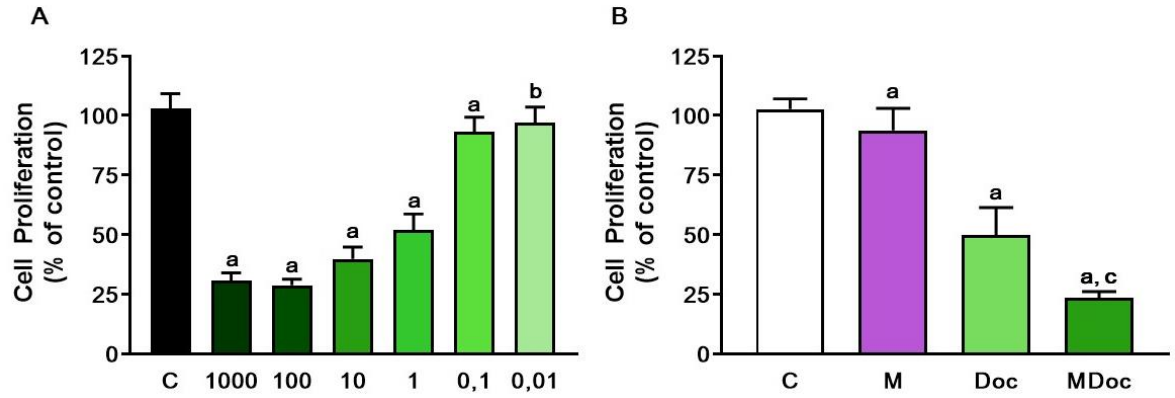


Figure 6. ***Effect of melatonin pre-treatment and docetaxel on MCF-7 cell proliferation. (A)*** *Cell proliferation measured at different docetaxel doses ranging from 1000 to 0.01 nM after 6 days of incubation* ***(B)*** *Effects of melatonin-pre-treatment and/or docetaxel (1 nM) on cell proliferation after 6 days of culture. Data are expressed as percentage of control cells (mean ± SEM). a, p<0.001 vs. C; b, p<0.01 vs. C; c, p<0.001 vs. Doc.* ***C:*** *Control,* ***M:*** *1 nM melatonin pre-treated cells,* ***Doc:*** *1 nM Docetaxel,* ***MDoc:*** *Melatonin and docetaxel.*

The other chemotherapeutic agent used in this work was doxorubicin. Similarly to the taxane, doxorubicin inhibited MCF-7 cell proliferation at different doses, showing the greatest inhibition at doses over 100 nM (**Figure 7A**). Once again, melatonin exerts its well-known anti-proliferative actions decreasing cell proliferation. When this hormone was combined with the anthracycline, both compounds potentiate their anti-proliferative effects, reaching the greatest inhibition (88%) compared with non-treated control cells (**Figure 7B**).

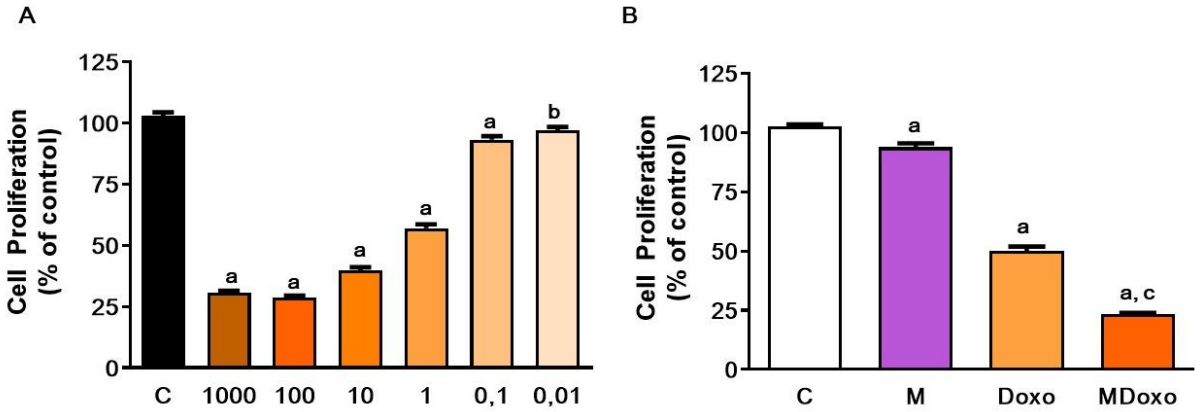


Figure 7. ***Effect of melatonin pre-treatment and doxorubicin on MCF-7 cell proliferation. (A)*** *Cell proliferation measured at different doxorubicin doses ranging from 1000 to 0.01 nM after 6 days of incubation.* ***(B)*** *Effects of melatonin-pre-treatment and/or doxorubicin (1 nM) on cell proliferation after 6 days of culture. Data are expressed as percentage of control cells (mean ± SEM). a, p<0.001 vs. C; b, p<0.01 vs. C; c, p<0.001 vs. Doxo.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***Doxo:*** *1 nM Doxorrubicin;* ***MDoxo****: Melatonin and doxorubicin.*

Effects of chemotherapeutic agents and melatonin on the expression of breast cancer-related miRNAs

To evaluate the effects of melatonin and chemotherapeutic agents in miRNA expression patterns in breast cancer cells we used the same Human Breast Cancer microarray tecnhnology previously mentioned. In this case, the chemotherapeutic agents were used at higher doses (1 µM) since the treatments were maintained only for 4 hours.

Establishing a fold-change of ±2, melatonin pre-treatment showed a downregulation of 26 miRNAs and an upregulation of 7 miRNAs when compared to control cells (**Figure 8A**). When docetaxel was administered alone, the expresion of 39 miRNAs (32 downregulated and 7 upregulated) was modified as displayed in **Figure 8B**. Finally, when both treatments were combined, 8 miRNAs were downregulated and 2 upregulated compared to control cells (**Figure 8C**). Relative quantity (ΔC_T) of selected miRNAs was plotted in **Figure 8D**. Validation of these data was done by specific qPCR for each miRNA.

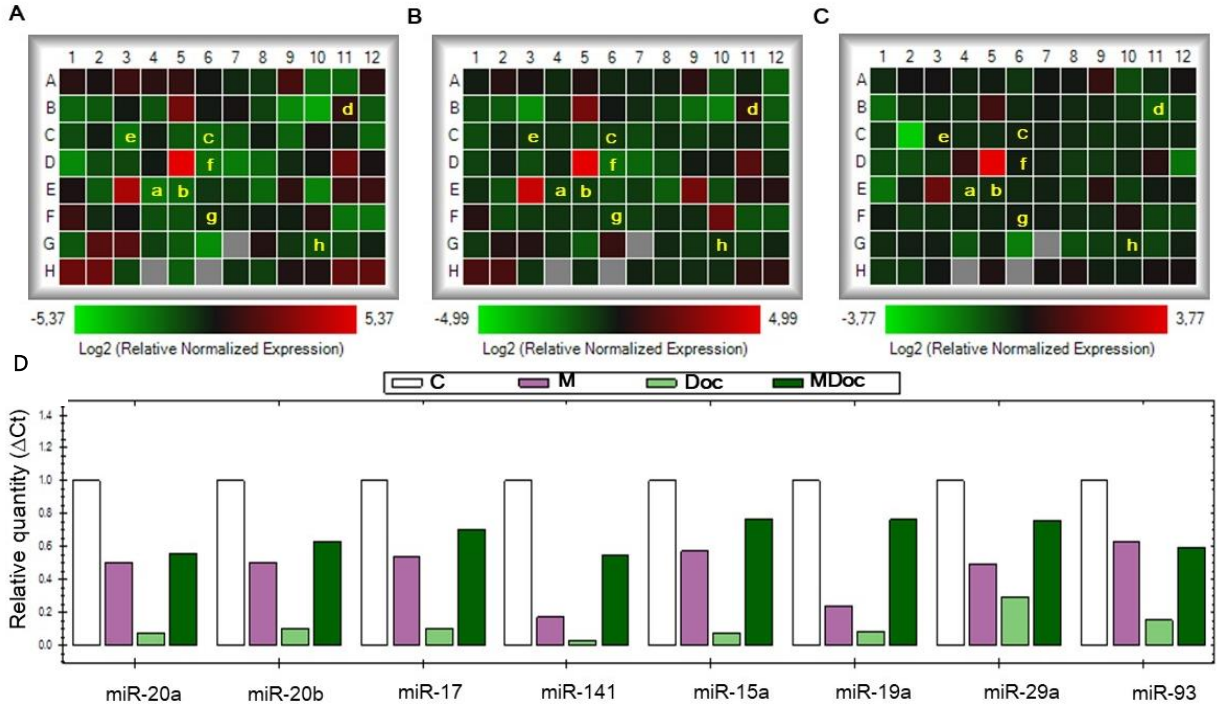


Figure 8. ***Effects of melatonin and docetaxel on breast cancer miRNAs expression microarray****. Cells were pre-treated with melatonin (1 nM) for one week and/or docetaxel (1 µM). After 4h, total RNA was extracted, reverse transcribed and used for qPCR analysis using miRNA Human Breast Cancer microarray (MIHS-109ZA). Panels* ***A-C*** *show heatmaps of relative normalized expression between different treatments* ***(A)*** *M vs C;* ***(B)*** *Doc vs C;* ***(C)*** *MDoc vs C.* ***(D)*** *Relative quantity (ΔC_T) of selected miRNAs bar chart. a: miR20a; b: miR-20b; c: miR-17; d: miR-141; e: miR-15a; f: miR-19a; g: miR29a; h: miR-93.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***Doc:*** *Docetaxel (1 µM);* ***MDoc****: Melatonin pre-treated and docetaxel.*

Results extracted from the microarray profiles were further validated by specific qPCR. When MCF-7 cells were pre-treated with 1 nM of melatonin, miRNA expression levels generally decreased, except from miR-93, miR-29a, miR-19a and miR-15a. Similar results were found when cells were treated with docetaxel, reducing the expression of miR-20a, miR-20b and miR-17. On one hand, addition of melatonin before the taxane was able to potentiate docetaxel effect on miR-20a and miR-20b. On the other hand, docetaxel administration significantly induced the expression of miR-141, miR-15a and miR-19a. However, when cells were treated with melatonin prior to docetaxel, this hormone was able to counteract these stimulatory effects of docetaxel (**Figure 9**).

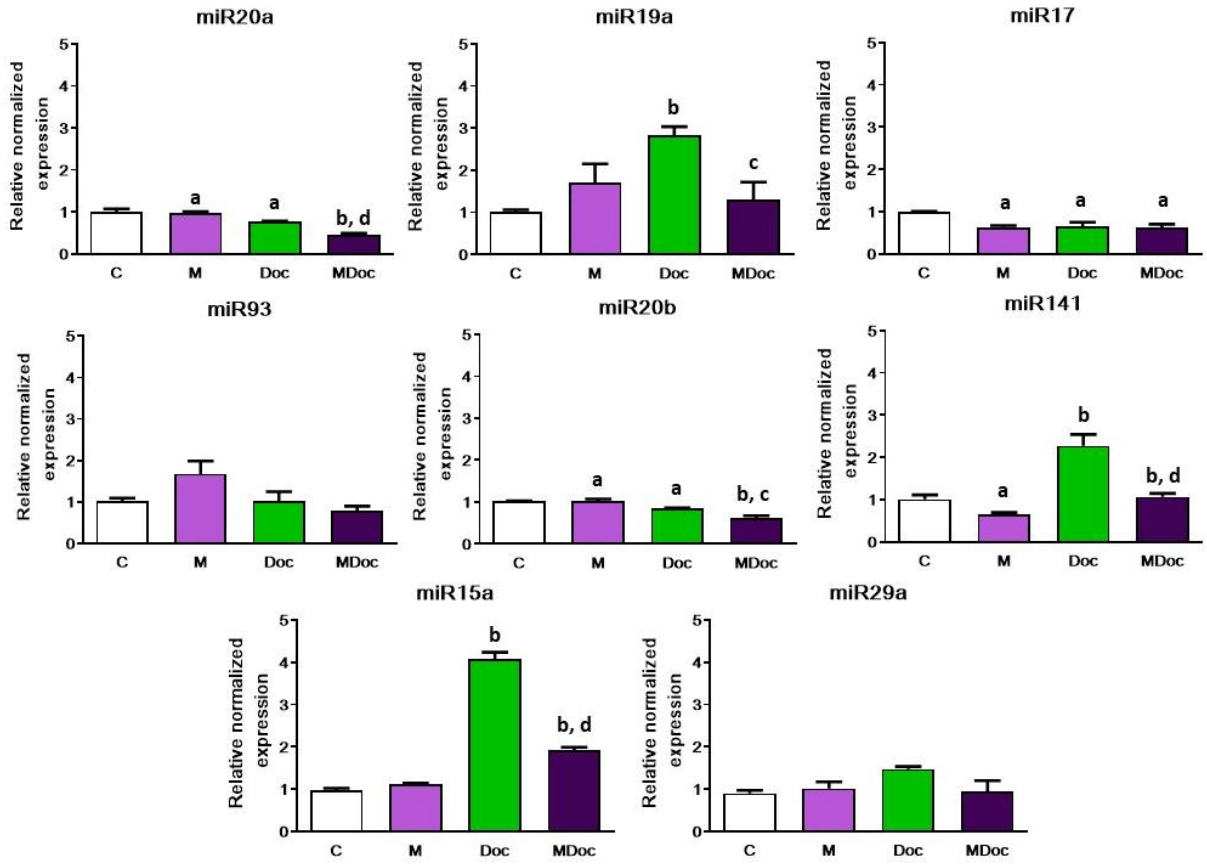


Figure 9. ***qPCR analysis of the specific miRNAs relative levels in MCF-7 cells.*** *Cells were treated with 1 nM melatonin and/or docetaxel (1µM), and after 4 h total RNA was isolated and reverse transcribed. cDNA was subjected to qPCR using miRNA specific primers. Data are expressed as a relative normalized expression compared to control cells (mean ± SEM). a, p<0.01 vs. C; b, p<0.001 vs. C; c, p<0.05 vs. Doc; d, p<0.01 vs. Doc.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***Doc:*** *Docetaxel (1 µM);* ***MDoc****: Melatonin pre-treated and docetaxel.*

Finally, we tested the effect of melatonin on miRNA expression changes induced by doxorubicin in MCF-7 cells. The most important changes were observed when the anthracyclin was added alone to the culture media, inducing a downregulation of 40 miRNAs and an upregulation of 6 miRNAs (**Figure 10B**). When cells were pre-treated with melatonin for one week before doxorubicin administration, only 17 miRNAs were modified (13 were downregulated and 4 upregulated) compared to non-radiated control cells (**Figure 10C**). Relative quantity (ΔC_T) of selected miRNAs from previous experiments of this work was plotted in **Figure 10D** in order to compare the effects between different treatments.

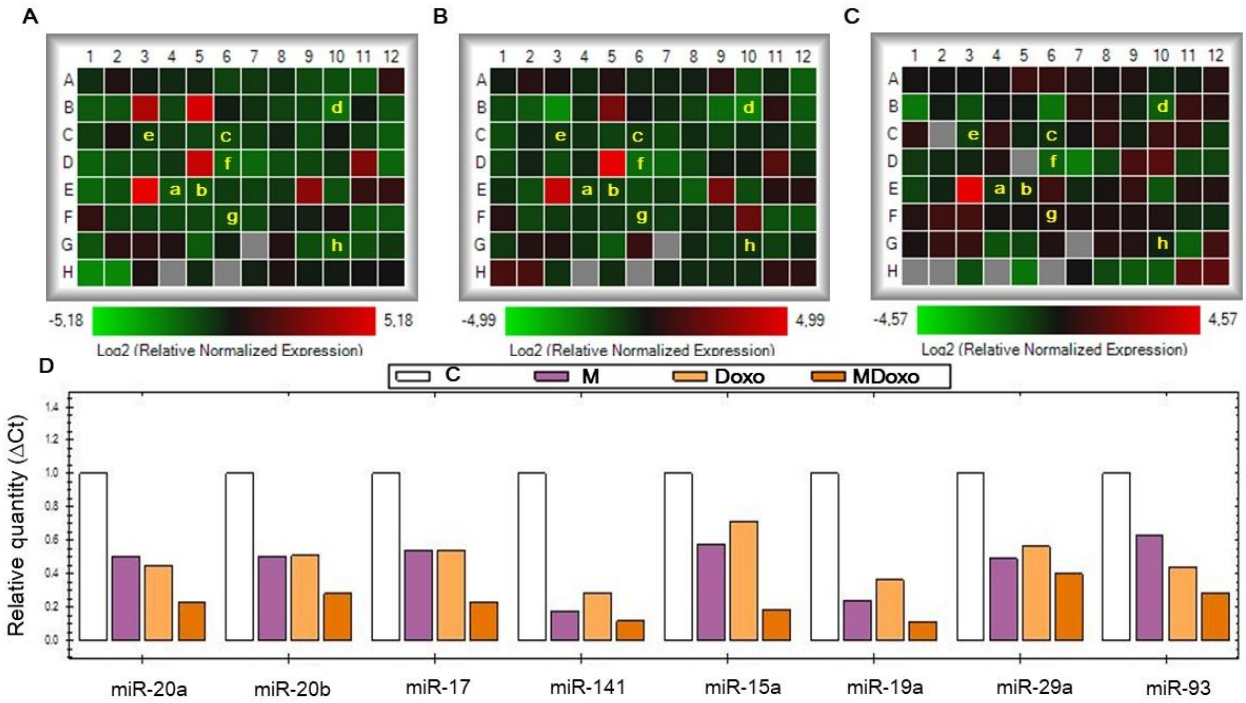


Figure 10. ***Effects of melatonin and doxorubicin on breast cancer miRNAs expression microarray****. Cells were pre-treated with melatonin (1 nM) for one week and/or doxorubicin (1 µM). After 4h, total RNA was extracted, reverse transcribed and used for qPCR analysis using miRNA Human Breast Cancer microarray (MIHS-109ZA). Panels* ***A-C*** *show heatmaps of relative normalized expression between different treatments* ***(A)*** *M vs C;* ***(B)*** *Doxo vs C;* ***(C)*** *MDoxo vs C.* ***(D)*** *Relative quantity (*ΔC_T*) of selected miRNAs bar chart. a: miR20a; b: miR-20b; c: miR-17; d: miR-141; e: miR-15a; f: miR-19a; g: miR29a; h: miR-93.* ***C:*** *Control;* ***M:*** *1 nM melatonin pre-treated cells;* ***Doxo:*** *Doxorubicin (1 µM);* ***MDoxo****: Melatonin pre-treated and doxorubicin.*

DISCUSSION

Potential of melatonin as an adjuvant molecule for radiotherapy treatment

In hormonal-dependent breast cancer, endocrine therapy is often combined with radiotherapy in order to improve the tumour response to breast cancer treatment (Zhong *et al*., 2013). Besides, pre-clinical models have shown that combination of aromatase inhibitors with radiation can enhance cytotoxicity and tumour cell death (Azria *et al.*, 2004).

Melatonin, the main product of the pineal gland, has been widely reported as an anti-tumoral and anti-aromatase hormone both *in vivo* and *in vitro* (Martínez-Campa *et al.*, 2017). Moreover, this indolamine has been related with radioprotective and radiosensitising properties trough modulation of enzymes involved in estrogen biosynthesis, inhibition of cell proliferation through p53 upregulation, modulation of DNA repair mechanisms or induction of cell-cycle arrest and apoptosis (Alonso-González *et al.*, 2020; Griffin & Marignol, 2018). Furthermore, melatonin exerts anti-angiogenic actions and neutralize proangiogenic effects induced by ionizing radiation (González-González *et al.*, 2019). In summary, all these properties point at this indolamine as a promising agent to be used as an adjuvant to breast cancer conventional treatments.

Irradiation of mammary glands induces changes that lead to the reduction of cell proliferation, proving its effectiveness as a breast cancer treatment (González-González *et al.*, 2019). As supported in our project, literature indicates that radiation inhibits cell proliferation on MCF-7 cells (Kong *et al*., 2020; Alonso-González *et al.*, 2015). Initially, in this work we wanted to evaluate the capability of melatonin to sensitize MCF-7 breast cancer cells to ionizing radiation. Hence, MCF-7 cells pre-treated with melatonin at physiological concentrations prior to radiation showed a significant reduction on cell proliferation, enhancing the anti-proliferative effects of ionizing radiation.

Furthermore, melatonin relationship with breast cancer does not only involve cell proliferation but many other pathological processes impacting cell adhesion, migration, invasion and apoptosis, among others. Recent knowledge has attributed some of these effects to melatonin's interaction with microRNAs (Kong *et al*., 2020; Farhood *et al*., 2019). MicroRNAs, known as miRNAs, are small non-coding molecules that take part in several cellular pathways, some of them with biological relevance for breast cancer. Many literature has reported melatonin's capability of altering transcription factors, kinases, tumour suppressor genes and oncogenes via differential expression of miRNAs (Chuffa *et al.*, 2020).

To study the effects of melatonin and radiation on miRNAs expression patterns in MCF-7 cells, we used a miRNA Human Breast Cancer microarray (MIHS-109ZA, Qiagen, USA) that contains 84 mature miRNA whose expression is known to be altered in breast cancer. When cells were pre-treated for one week with melatonin at physiological concentrations or radiated at a dose of 8 Gy, 33 out of the 84 total miRNAs were modified. Interestingly, the most significant changes were observed when both treatments were combined (melatonin pre-treatment and radiation), observing changes in 44 miRNAs.

As the PCR array contains only one well for each of the miRNAs studied, it was neccesary to corroborate these results with speficic qPCRs. Due to their relevance in breast cancer pathogenesis, we selected a specific set of eight miRNAs to validate this data: miR-20b, miR-93, miR-17, miR-20a, miR-19a, miR-29a, miR-15a, miR-19a and miR-141. Most of these miRNAs belong to highly conserved miRNAs clusters whose expression is increased in a wide spectrum of cancers, including breast cancer. The miR-17-92 cluster encodes for six mature miRNAs, comprising miR-17, miR-20a and miR-19a as the most oncogenic ones. Besides, there are two paralogue gene clusters, miR-106a-363 and miR-106b-25, which encodes for miR-20b, miR-93 and miR-19a (Fang *et al.*, 2017). These miRNA clusters have demonstrated a cooperative function in regulating targeted gene expression.

The miR-17-92 cluster was found to be overexpressed in breast tumours. Although there are controversial findings on its role in breast cancer pathogenesis, this cluster is considered to have a oncogenic behaviour (Fang *et al.*, 2017). For example, miR-20a and miR-19a, members of this cluster, are mainly involved in PTEN regulation, which is a key tumour suppressor gene. Concretely, PTEN encodes for a phosphatase which downregulates PI3K/AKT signalling pathway, related with cell motility, invasion, proliferation and survival (Carbognin *et al.* 2019; Hamam *et al.* 2017). In our work, both melatonin and radiation treatments significantly reduced miR-20a and miR-19a relative expression. Besides, when melatonin was added to the culture media before radiation, the inhibition was even higher.

Regarding miR-17, radiation alone induced a significant upregulation which correlates with c-Myc overexpression and PTEN suppression (Nurzadeh *et al.*, 2021). However, this undesirable radiation effect was counteracted by melatonin pre-treatment. Since there are many data in the previous literature that points to a correlation between miR-17-92 cluster overexpression and poor clinical state (Nurzadeh *et al.*, 2021), the ability of melatonin to diminish its expression may be considered as a positive beneficial effect in breast cancer.

Other miRNAs (miR-93 and miR-29a) are related to angiogenic and metastatic processes. Hence, both miRNAs stimulate endothelial cell growth and metastasis in breast cancer (Fang *et al.*, 2012) and their expression correlates with a migratory and invasive phenotype on MCF-7 cells (Singh & Mo, 2013). In our results, both miRNAs were underexpressed in melatonin pre-treated and radiated groups when compared to non-treated cells, and, once again both treatments were found to cooperate inducing the highest inhibition.

miR-20b, a member of miR-106A-363 cluster, is also related with PI3K/AKT pathway. Specifically, overexpression of this miRNA induces Akt activation which in turn supresses PTEN and p53 proteins, thus promoting tumorigenic processes (Fang *et al.*, 2017). Again, melatonin seems to exert a protective effect, since the inhibition of miR-20b was higher when this indolamine was added previously to the ionizing radiation treatment.

miR-141 is a member of miR-200 family, a key regulator of EMT transition through E-cadherin expression levels, acting as a tumour suppressor. However, recent data reports that this miRNA can either

act as an oncomiR when it is upregulated depending on the biological process (Nurzadeh *et al.*, 2021; Kaban *et al.*, 2019; Loh *et al.*, 2019). Our study reveals an upregulation of this miRNA when cells were irradiated and a downregulation when melatonin was administered before radiation. As intriguing as it may be, there is controversial literature regarding its role in metastatic processes. As MCF-7 cells are a non-metastatic cell line, therefore, this miRNA could be underexpressed in this particular case. Moreover, it is important to add that some studies affirmed melatonin modulated miRNA expression, however this does necessarily mean these miRNAs are causative of tumoral processes (Ferreira *et al.*, 2020).

Finally, miR-15a is known for its tumour suppressor role and its overexpression is related with cyclin E1 inhibition, which is primarily related with G1-S transition (Loh *et al.*, 2019). Therefore, overexpression of miR-15a has beneficial effects for breast cancer progression. In our results, melatonin reduced the overexpressed miR-15a levels induced by radiation. However, recent studies indicate that miR-15a is downregulated in breast cancer (Luo *et al.*, 2013) suggesting that melatonin can act as a regulator of deleterious overexpression of this miRNA, which can affect cytotoxicity. miRNA regulations and implications are summarized in **Table 1**.

Table 1. ***MiRNA expression patterns under radiotherapy and melatonin treatment and related molecular pathways.***

miRNA	Radiation	Radiation and melatonin	Related pathway	Associated event
miR-20a	Downregulated	Downregulated	PI3K/AKT/mTOR	Invasion, proliferation and survival
miR-19a	Downregulated	Downregulated	PI3K/AKT/mTOR	Invasion, proliferation and survival
miR-17	Upregulated	Downregulated	PI3K/AKT/mTOR	Promotes invasion and metastasis
miR-93	Downregulated	Downregulated	PI3K/AKT/mTOR	Promotes invasion and metastasis
miR-20b	Downregulated	Downregulated	PI3K/AKT/mTOR	Cell growth, proliferation and apoptosis
miR-141	Upregulated	Downregulated	Wnt/β-catenin	Regulates EMT and invasion
miR-15a	Upregulated	Upregulated	Cyclin E1, E2F7	Anti-proliferative, G1-S cell cycle arrest
miR-29a	Downregulated	Downregulated	Akt3, VEGF, c-MYC	Regulates EMT and metastasis

We next determined the ability of melatonin to act as a modulator of some key kinases and their phosphorylation levels. Therefore, we focused our attention on biologically relevant signalling pathways involved in breast cancer, specifically studying Akt (PI3K/AKT) and p70S6 kinase (mTOR). In a proteome profiler assay, we found that melatonin treatment before radiation induced an inhibition on Akt and p70S6 kinases phosphorylation compared to radiated cells, suggesting a melatonin protective effect, as these are both tumour promoting kinases (Kong *et al.*, 2020).

Phosphorylation of Akt correlates with poor prognosis since when is active sends pro-survival signals to the cell (Carbognin *et al.*, 2019). Moreover, activated Akt leads to mTOR activation, that activates, in turn, p70S6 kinase, increasing protein synthesis and cell proliferation (Kurgan *et al.*, 2017). Suppression of PTEN activates the AKT/mTOR/p70S6K signaling axis, leading to a proliferative phenotype. When we tested Akt and p70S6K protein levels by western-blot, we found a modest stimulation on total Akt and p70S6K protein levels in the radiated cells, but this deleterious effect was counteracted by melatonin pre-treatment. However, we did not observe significant changes in the levels of their phosphorylated

versions when cells were irradiated. Nevertheless, melatonin administration before radiation treatment was able once again to diminish p-Akt and p-p70S6 levels, proving its radio-sensitizing effects.

In summary, the combination of melatonin with radiation in breast cancer cells has positive results in breast cancer treatment by inhibiting cell proliferation, decreasing AKT/mTOR/p70S6K signaling pathway and polarizing miRNAs expression levels towards a less proliferative and invasive phenotype. All these effects make us consider melatonin as a promising adjuvant agent to radiotherapy.

Potential of melatonin as an adjuvant molecule for chemotherapy treatment

Chemotherapy is traditionally one of the most common breast cancer treatments, despite its devastating side effects (Barzaman *et al.*, 2020). The two chemotherapeutic agents used in this work are docetaxel and doxorubicin. The first one is a taxane that acts stabilizing microtubules through tubulin binding, consequently preventing cell division. The second one is an anthracycline that works by intercalation between double DNA helix, inhibiting DNA dependent DNA and RNA polymerases, interrupting DNA replication as a result (Saloustros *et al.*, 2008; Czeczuga-Semeniuk *et al.*, 2004).

To prove potential action of melatonin as an adjuvant molecule for chemotherapeutic treatments, one of the first goals of this part of the work was to perform cell proliferation studies on MCF-7 cells. Firstly, we corroborate the anti-proliferative effect of the taxane and the anthracycline, founding a dose-dependent inhibition. Even sub-pharmacological doses of these drugs showed a significant inhibition in comparison to control cells. Moreover, administration of physiological doses of melatonin potentiated the inhibition of cell proliferation triggered by docetaxel or doxorubicin. This synergistic effect may be due to addition of melatonin's effect on G_1-S transition delay, reducing the number of cells present in the S phase (Maroufi *et al.*, 2020; Alonso-González *et al.* 2017). These results point to melatonin as an enhancer of chemotherapeutic agents' effects, even when they are used at sub-pharmacological doses.

Once established that melatonin enhanced the anti-proliferative effects of these chemotherapy agents, we next decided to evaluate whether this indolamine was able to modify chemotherapy-induced changes on miRNAs expression patterns. As mentioned before, for this purpose we employed a miRNA Human Breast Cancer microarray (MIHS-109ZA, Qiagen, USA).

Regarding miRNA expression under chemotherapeutic treatment, our results demonstrated a change in miRNA expression levels in MCF-7 cells treated with chemotherapy and melatonin, either alone or in combination. The analysis revealed a general reduction of miRNA set in docetaxel-treated cells when compared to their respective non-treated control. However, when melatonin was added prior to the taxane, miRNA expression, despite remaining lower than non-treated cells, displayed higher levels than in docetaxel treatment. On the contrary, doxorubicin treated cells, exhibited higher levels than doxorubicin and melatonin pre-treated cells. Due to this high variability in miRNA microarrays, validation by specific qPCR was required to confirm this experimental data.

Specific qPCRs disputed previous results regarding docetaxel and melatonin pre-treatment. In our results miR-20a, miR-17 and miR-19a which are members of miR-17/92 cluster, are generally downregulated after docetaxel treatment, except for miR-19a, whose expression levels are increased in respect to non-treated cells. This may be due to multidrug resistance (MDR), as an upregulation of this miRNA has been observed in chemoresistant cancers (Wu *et al.*, 2014).

Despite of the effects observed in the microarray, melatonin addition to the culture media before docetaxel induced a general inhibition on the expression altered by the taxane. Since this cluster is mainly involved in PI3K/AKT signalling pathway, it is positive that these miRNAs are downregulated in MCF-7 breast cancer cells treated with melatonin, pointing to a protective effect in a way that fits with its anti-tumoral actions (Carbognin *et al.*, 2019). This further proves the positive effect of melatonin in sensitizing breast cancer cells towards conventional treatments.

In relation to miR-20b, a member of miR-106A-363 cluster, both docetaxel and melatonin treatments showed a downregulation. Consistently with the previous results, implication of this miRNA in Akt activation and PTEN and p53 regulation, demonstrates the positive effect of melatonin in the polarization towards apoptotic processes (Fang *et al.*, 2017).

Regarding miR-141, key regulator of EMT transition, we found overexpressed levels when cells were treated with docetaxel but a downregulation when melatonin was previously added to the culture media. This miRNA family overexpression has also been linked to chemotherapy resistance (Loh *et al.*, 2019). In agreement with recent published works, our results were consistent as miR-141 expression is correlated with docetaxel resistance since inhibition of miR-141 enhanced response to docetaxel, inhibiting cell viability and increasing apoptosis in lung cancer (Wang *et al.*, 2017). For that reason, melatonin's ability to diminish docetaxel-induced miR-141 expression levels indicate a positive role of this indolamine to avoid chemotherapy resistance (Ferreira *et al.*, 2020).

Although the beneficial effects of miR-15a upregulation had been stated before, regarding chemotherapeutic treatment, its upregulation may have a different outcome. Literature has acknowledged the dual role of this miRNA as an oncogene and tumour suppressor depending on tissue microenvironmental characteristics (Krishnan, 2016). What can be said is that melatonin regulates this miRNA expression, that can be polarized by chemotherapeutic drugs used to treat breast cancer. This information is gathered in **Table 2**.

It is important to consider that despite new findings may arise from the promising results regarding breast cancer molecular machinery and specifically to miRNA regulation, it is possible that melatonin is exerting its effects via subtle modulations of miRNA clusters and families and not a specific miRNA (Ferreira *et al.*, 2020; Loh *et al.*, 2019). Therefore, these results must be considered carefully as they might be part of a more complex and profound network that requires thorough evaluation.

Table 2. ***Expression patterns of miRNAs under docetaxel and melatonin treatment and related molecular pathways.***

miRNA	Docetaxel	Docetaxel and melatonin	Related pathway	Associated event
miR-20a	Downregulated	Downregulated	PI3K/AKT/mTOR	Invasion, proliferation and survival
miR-19a	Upregulated	Downregulated	PI3K/AKT/mTOR	Invasion, proliferation and docetaxel resistance
miR-17	Downregulated	Downregulated	PI3K/AKT/mTOR	Promotes invasion and metastasis
miR-93	No effect	No effect	PI3K/AKT/mTOR	Promotes invasion and metastasis
miR-20b	Downregulated	Downregulated	PI3K/AKT/mTOR	Cell growth, proliferation and apoptosis
miR-141	Upregulated	Downregulated	Wnt/β-catenin	Regulates EMT, invasion and chemoresistance
miR-15a	Upregulated	Upregulated	Cyclin E1, E2F7	Anti-proliferative, G1-S cell cycle arrest
miR-29a	No effect	No effect	Akt3, VEGF, c-MYC	Regulates EMT, metastasis and docetaxel resistance

In summary, present evidences place melatonin as a potential adjuvant for breast cancer conventional treatments. It is not a surprise after manifesting its oncostatic properties on estrogen-dependent breast cancer cell lines, restoring different molecular pathways that seem to be altered by cancer. In spite of this, not much attention has been paid to the intriguing molecular mechanisms underlying melatonin regulatory effects. Moreover, miRNAs implication in these regulatory actions is critical, as they represent specific and biologically relevant targets for this disease (**Figure 1**). This, added to the fact that miRNAs are promising molecules in personalized medical treatments, opens up a myriad of possibilities to tackle two major problems that remain unsolved in breast cancer: resistance and side effects.

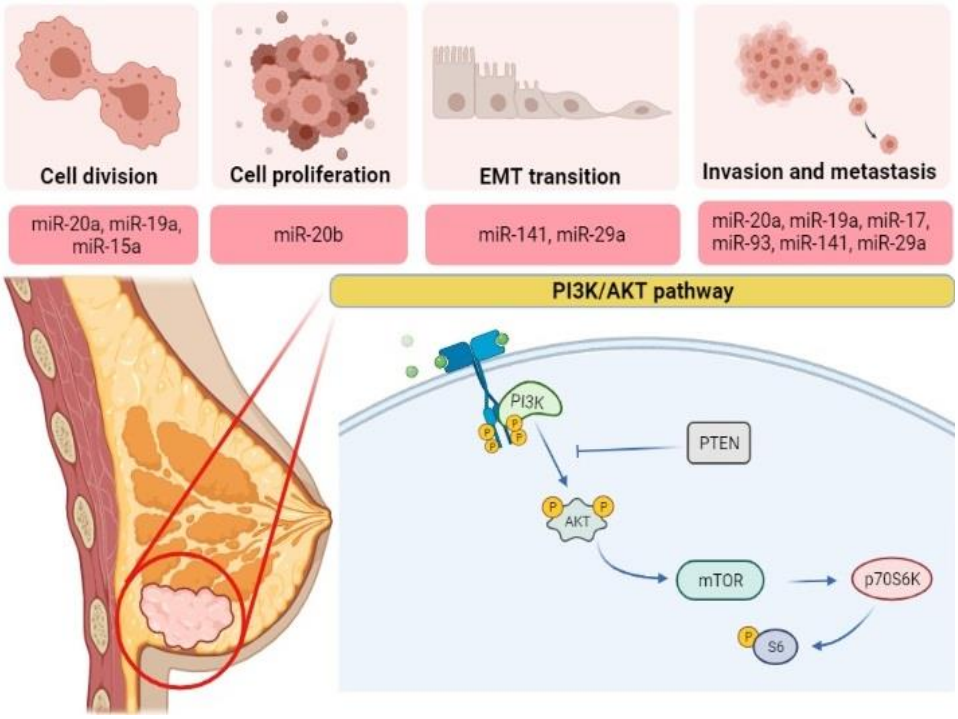


Figure 4. ***Breast cancer pathological processes and implication of selected miRNAs.*** *Diagram of PI3K/AKT pathway signalling cascade is shown above.*

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, physiological doses of melatonin enhance the antiproliferative effects of ionizing radiation and chemotherapeutic agents (docetaxel and doxorubicin) on MCF-7 breast cancer cells.

Ionizing radiation and chemotherapy induce changes in breast cancer-related miRNA expression in MCF-7 cells and melatonin added prior to these treatments modulates these changes towards a non-cancerous behaviour.

Phosphorylation levels of two of the main proteins participating in PI3K/AKT/mTOR signalling axis (Akt and p70S6K) are reduced after irradiation of MCF-7 cells, being this effect potentiated by melatonin pre-treatment before ionizing radiation.

To summarize, conventional breast cancer treatments have beneficial effects on MCF-7 regarding cancer progression. These effects are enhanced by melatonin, pointing at a synergistic effect when treatments are combined with this indolamine, sensitizing breast cancer cells towards conventional treatments.

According to these findings, future research will be focused on validating doxorubicin preliminary results on miRNA expression patterns by specific qPCR. Other objective will be to establish the functional implication of selected miRNAs on gene expression changes and cell biology processes, transfecting MCF-7 cells to overexpress or to silence the selected miRNAs and studying their modulation on cell proliferation, migration or invasion.

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